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14. ABSTRACT

Project 1:

Dilated cardiomyopathy affects approximately 1 in 2,500 individuals in the United States and is the 3rd most common cause of heart failure and the most frequent cause of heart transplantation. Patients that suffer from various muscle diseases, including Duchenne muscular dystrophy (DMD), develop progressive cardiomyopathy. Cellular cardiomyoplasty, which involves the transplantation of exogenous cells into the heart, is a possible approach by which to repair diseased or injured myocardium and improve cardiac function. Though there are a number of drugs prescribed to treat dilated cardiomyopathy, there is no cure and individuals eventually require a heart transplant; therefore the use of cardiomyoplasty to repair the hearts of individuals suffering from cardiomyopathy could possibly be an effective alternative to heart transplantation.

Technical Objective #1: To investigate the effect of cell survival, proliferation, and differentiation on the regeneration/repair capacity of various human MDSC populations implanted into the heart of mdx/SCID mice.

Technical Objective #2: To investigate the role that angiogenesis plays in the regeneration/repair capacity of human MDSCs injected into the hearts of mdx/SCID mice.

Project 2:

This project will determine the extent to which novel sources of hepatocytes can be used for regeneration and repair of injuries to the liver and liver failure. A more complete understanding of the extent to which donor liver cells can be resuscitated from non-traditional sources and expanded for application to reduce liver injury and toxin and/or cancer risk should enhance the number of areas where hepatic stem cell transplantation might be effectively applied.

Technical Objective #1: To characterize and expand hepatocytes from patients with cirrhosis and end-stage liver disease in immune deficient hosts whose livers permit extensive repopulation with donor cells.

Technical Objective #2: To determine the extent to which transplantation with human hepatocytes can reverse hepatic failure in a clinically relevant non-human primate model of this process.

15. SUBJECT TERMS

Project 1: Duchenne muscular dystrophy, Cardiomyopathy, Cell Therapy, Cellular cardiomyoplasty, Pericytes, myo-endothelial cells, Angiogenesis,

Project 2: Liver disease, Cirrhosis, Hepatocyte isolation, Hepatocyte transplantation, Induced Pluripotent Stem (iPS) cells

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Sub-project 1: Muscle Stem Cell Therapy for the Treatment of

DMD Associated Cardiomyopathy

PI: Johnny Huard

INTRODUCTION:

Cardiomyopathy is a serious disease of the heart muscle that can lead to congestive heart failure, a condition in which the heart can no longer effectively pump blood. Dilated cardiomyopathy affects approximately 1 in 2,500 individuals in the United States and is the 3rd most common cause of heart failure and the most frequent cause of heart transplantation. Patients that suffer from some diseases of the cytoskeleton, such as the progressive muscular dystrophies, often develop cardiomyopathy. Duchenne muscular dystrophy (DMD), one of the progressive muscular dystrophies, is an X-linked muscle disease caused by mutations in the dystrophin gene. This devastating disease is characterized by progressive muscle weakness due to a lack of dystrophin expression at the sarcolemma of muscle fibers. DMD patients usually develop symptoms of dilated cardiomyopathy in their early teens, and these symptoms steadily progress with age. This dilated cardiomyopathy is characterized by an enlarged ventricular chamber, wall thinning, and systolic dysfunction. Histological examination of a human DMD cardiomyopathic heart reveals fibrosis, degeneration, and fatty infiltration. The lack of dystrophin has consequently been linked to the cardiomyopathy that develops in DMD. In the hearts of mdx, dystrophin-deficient, mice (a murine model of DMD), especially in aged mdx mice, fibrosis and degeneration of the myocardium are evident upon histological examination. Because the mammalian heart possesses only a limited capacity to regenerate new cardiac muscle after injury and disease, noncontractile scar tissue eventually replaces the injured and diseased myocardium. Cellular cardiomyoplasty, which involves the transplantation of exogenous cells into the heart, is a possible approach by which to repair diseased or injured myocardium and improve cardiac function. Though there are a number of drugs prescribed to treat dilated cardiomyopathy, there is no cure and individuals eventually require a heart transplant; therefore the use of cardiomyoplasty to repair the hearts of individuals suffering from cardiomyopathy could possibly be an effective alternative to heart transplantation.

Technical Objectives

Technical Objective #1: To investigate the effect of cell survival, proliferation, and differentiation on the regeneration/repair capacity of various human muscle derived cell populations implanted into the heart of aged mdx/SCID mice and/or dystrophin/utrophin double knock-out mice.

Hypothesis: *After implantation into the heart, the human muscle derived cells abilities to survive and undergo long-term proliferation play a role in their efficient regeneration and repair of diseased cardiac tissue.*

Technical Objective #2: To investigate the role that angiogenesis plays in the regeneration/repair capacity of human muscle derived cell populations injected into the hearts of aged mdx/SCID mice and/or dystrophin/utrophin double knock-out mice.

Hypothesis: *Enhancing angiogenesis will increase the regeneration/repair capacity of human muscle derived cells injected into the hearts of dystrophic mice.*

Technical Objective #3: To investigate the potential beneficial effect that creating a parabiotic pair between dystrophin/utrophin double knock-out (dKO) mice and normal mice might have on preventing the progression of cardiomyopathy in the dKO animals.

Hypothesis: *Parabiosis between dKO and normal mice will slow the progression of cardiomyopathy in the dKO mice.*

Technical Objective #4: To investigate the role that RhoA signaling plays in the cardiac dysfunction observed in dKO mice and determine if inactivating RhoA might have a beneficial effect on improving the dystrophic cardiac phenotype observed in the dKO mice
Hypothesis: *RhoA inactivation will improve the dystrophic cardiac phenotype observed in the dKO mice***.**

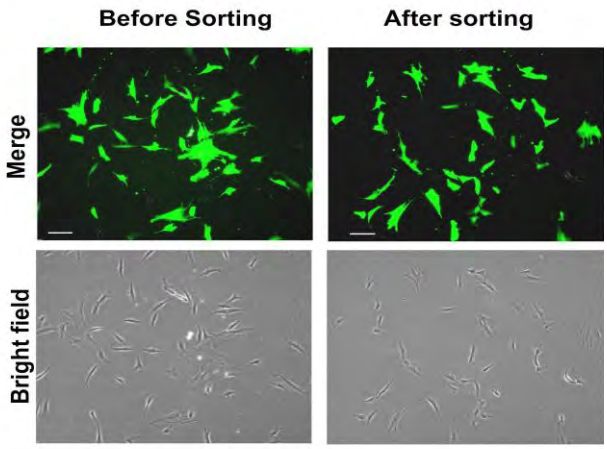
BODY:

Progress made from: 10-1-13 to 9-30-14

1) Lenti-GFP transduction of human muscle-derived stem cells

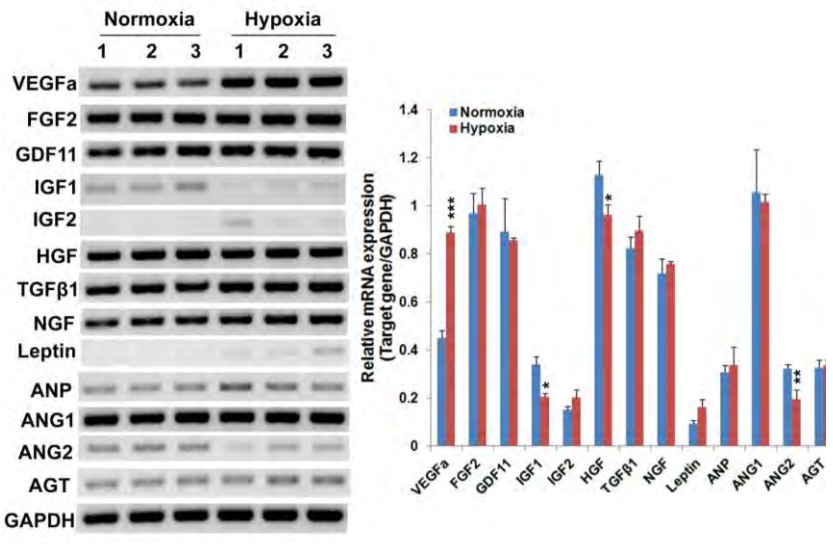
As outlined in our previous progress report human muscle derived stem cells (hMDSCs) were transduced with lenti-GFP at passage 10 using a 1:1 dilution of the lenti-GFP virus. The cells were further expanded and subjected to Fluorescence activated cytometry (FAC) sorting for GFP positivity. The GFP positivity is shown in **Figure 1.**

Figure 1



2) Effect of hypoxia on the gene expression of human muscle derived stem cells (hMDSCs)

Figure 2



Effects of hypoxia on the gene expression of lenti-GFP transduced hMDSCs. A. Eletrophoresis images of different genes. B. Quantification of gene expression after hypoxia treatment. *p<0.05,** p<0.01,***p<0.001.

Three populations of lenti-GFP transduced hMDSCs were cultured in proliferation medium that contains DMEM supplemented with 20% fetal bovine serum, 1% chicken embryo extract, and 1% penicillin/streptomycin. 5×10^4 cells were seeded in two 6-well plates and cultured in proliferation medium overnight. Cells were then rinsed with PBS 2 times and serum free DMEM added to the cells. One plate was cultured in 2.5% oxygen atmosphere and another plate was incubated in 20% normoxic incubator. After 24 hrs, the supernatant was collected for ELISA. The cells were rinsed with PBS 1 time, tripsinzed and the cell numbers were determined. The cell pellets were then lysed with 1ml Trizol (Invitrogen) and total RNA was extracted

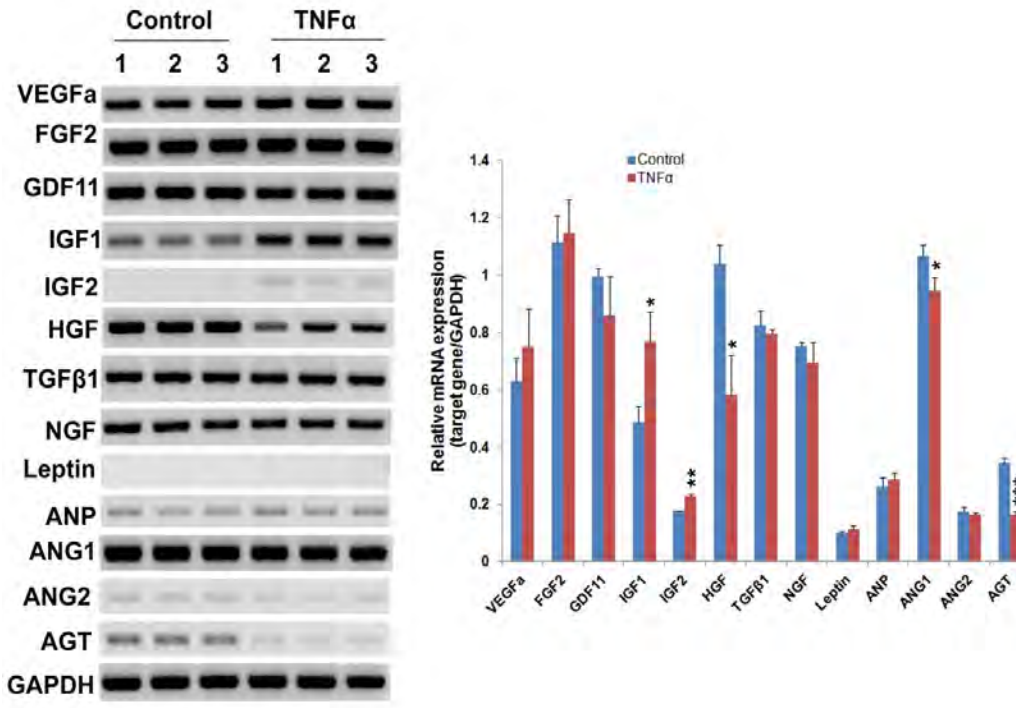
according to the manufacturer's instructions. 650ng total RNA was used for cDNA synthesis using a Maxima cDNA Synthesis kit (Fisher Scientific). Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was performed using a Gotaq PCR kit (Promega). The experiment was repeated in triplicate.

The results showed that under hypoxia culture conditions, vascular endothelial cell growth factor (VEGFa) expression was significantly upregulated in the hMDSCs, while insulin like-growth factor-1(IGF-1), hepatocyte growth factor(HGF),and angiopoietin 2 (ANG2) were all down-regulated significantly. Fibroblast growth factor 2(FGF2), growth development factor 11(GDF11), transformation growth factor β1 (TGF- β1), nerve growth factor (NGF) and angiopoietin 1 (ANG1) were consistently being highly expressed under both hypoxic and normoxic conditions. Interestingly, leptin gene expression was induced under hypoxia although there was no statistical difference observed. No changes in any other gene expression were found (**Figures 2, A-B**).

3) Effect of TNFα treatment on hMDSC gene expression

Three populations of lenti-GFP transduced hMDSCs were cultured in proliferation medium that contained DMEM supplemented with 20% fetal bovine serum, 1% chicken embryo extract, and 1% penicillin/streptomycin. 5×10^4 cells were seeded in 6-well plates and cultured in proliferation medium overnight. Cells were then rinsed with PBS for 2 times and serum free DMEM was added to the cultures that contained TNFα (100ng/ml) or just serum free DMEM as a control, each treatment was performed in triplicate. Cells were cultured for another 24 hrs. and the supernatant was then collected for ELISA. The cells were rinsed with PBS 1 time and then lysed with 1ml Trizol. The RNA extraction, cDNA synthesis and RT-PCR was performed using the same protocol stated above.

Figure 3

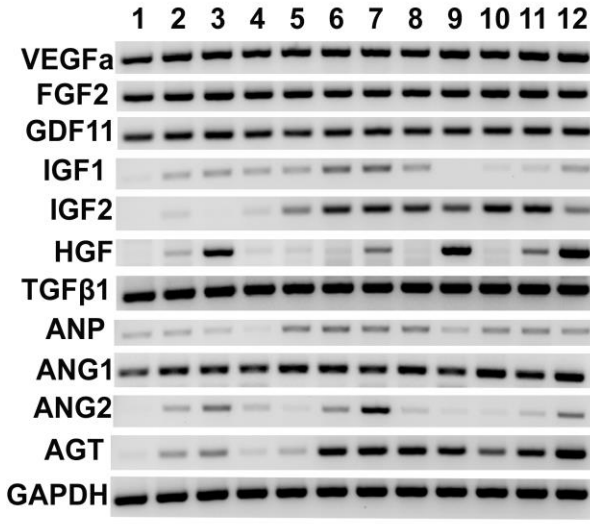


Effects of TNFα treatment on the expression of growth factors and cardiac functional gene. A. Eletrophoresis images of different gene expression. B. Quantification of relative mRNA expressions. *p<0.05, ** p<0.01,***p<0.001.

The results showed that TNFα treatment significantly increased IGF1 and IGF2 expression, but down regulated HGF, angiopoietin 1 and angiotensinogen (AGT). No effect on the expression of VEGFa, FGF2, GDF11, TGFβ1, NGF, leptin, atrial natriuretic peptide (ANP) or ANG2 was observed post-TNFα treatment (**Figure 3**).

4) Gene expression of 12 populations of cells isolated from human skeletal muscle and heart.

Figure 4



To further investigate the mechanism(s) responsible for the beneficial effect that hMDSCs have on aged heart repair, we compared the gene expression of hMDSCs with other populations of cells. Human MDSCs included: 23yo male (1), 20yo male (2), 31yo female (3), 76yo female (4), human PP1 fibroblasts included: 23yo male (5), 20yo male (6), 31yo female (7), 76yo female (8). We also compared these latter populations with human heart pericytes (9), human myoblasts (10), human cardiac fibroblasts (11), and human skeletal muscle pericytes (12).

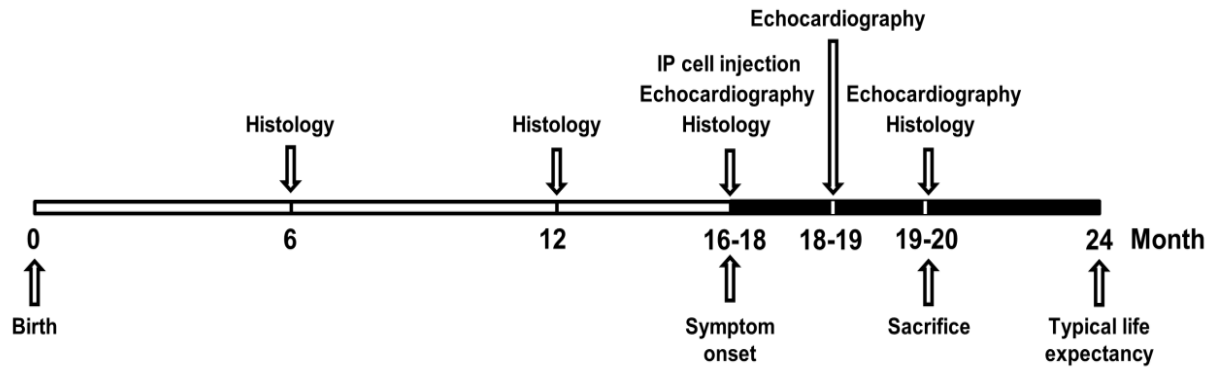
The cells were cultured and expanded and were harvested at different passages, rinsed with PBS and lysed with 1ml Trizol. 1ug total RNA was extracted and used for cDNA synthesis. RT-PCR was performed as stated above.

Our results indicated that the hMDSCs (populations 1-4) expressed similar high levels of VEGFa, FGF2, GDF11, TGFβ1, ANG1 as did the human muscle derived fibroblasts (populations 5-8), human heart pericytes (9), skeletal muscle myoblasts (10), cardiac fibroblasts (11), and skeletal muscle pericytes (12). There was differential expression of IGF1, IGF2, HGF, ANG2, AGT, and ANP by all the populations, though IGF2 and AGT showed fairly strong expression by the pp1 fibroblasts (populations 5-8) and by the human heart pericytes (9), human myoblasts (10), human cardiac fibroblasts (11), and human skeletal muscle pericytes (12), while the hMDSC populations showed almost no IGF2 expression (**Figure 4**). We are currently in the process of quantifying this data.

5) Intraperitoneal injection of hMDSCs in the dystrophic cardiomyopathy model

The current study design is illustrated in **Figure 5**. We used 26 senile mdx/SCID mice (16-18 month old) that displayed symptoms of DMD cardiomyopathy for the full-scale study. To investigate whether intraperitoneal injection of hMDSCs benefit dystrophic hearts, all mdx/SCID mice were randomly assigned to each group before the start of the study. The experimental group received a single injection of 1×10^6 GFP+ hMDSCs

Figure 5

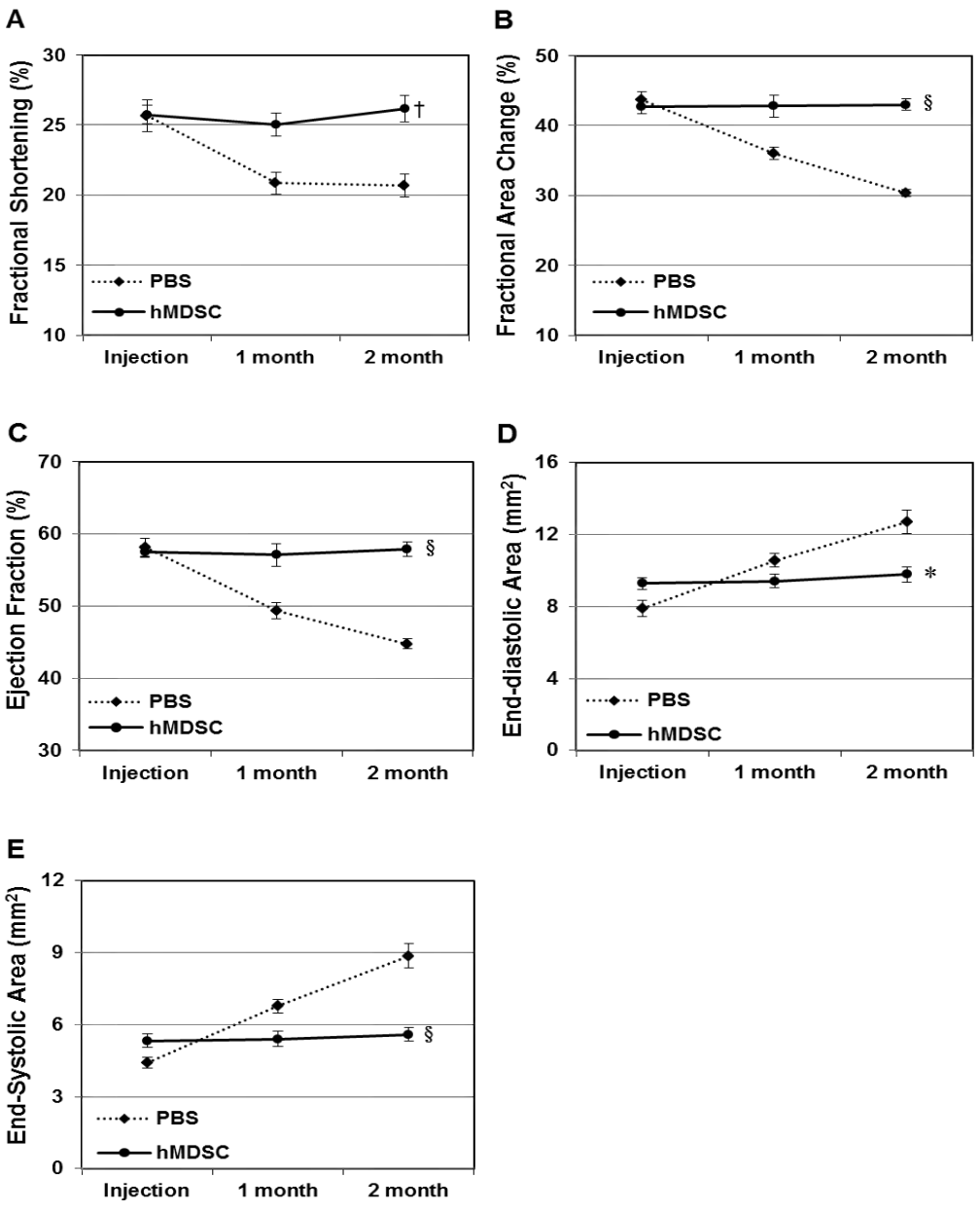


resuspended in 50 ul of PBS in the lower abdomen while control groups received an equal amount of GFP+ myoblasts or 50 ul PBS. Cardiac function was assessed by echocardiography performed repeatedly immediately before (baseline) and after injection at 1 and 2 months. Animals that died prior to 2 month post-injection were excluded from the study. Experiments for the injection of the first hMDSC population (donor #1, N=6) and PBS control (N=6) have been completed while experiments for the injection of the second hMDSC population (donor #2, N=6) and myoblast control (N=4) are ongoing.

6) Intraperitoneal administration of human muscle-derived stem cells prevent progressive failure of the aging dystrophic heart

Aging mdx/SCID mice (16-18 months old) were randomly assigned to each group before the intraperitoneal injection. The survival of animals receiving hMDSC or PBS treatment was monitored over the course of 2 months following the injection. Two control mice receiving PBS and one mouse receiving hMDSCs treatment died before the end of the experimental duration and were excluded from the study. Cardiac function was repeatedly assessed by echocardiography (N=6 mice per group) immediately before and at 1 and 2 months following the injection. Left ventricular (LV) contractility was evaluated by LV fraction shortening (LVFS), LV fractional area change (LVFAC, **Figure 6**), and LV ejection fraction (LVEF, **Figure 6**), whereas LV chamber size was measured by LV end-diastolic area (LVEDA, **Figure 6**) and end-systolic area (LVESA, **Figure 6**). No significant differences

Figure 6



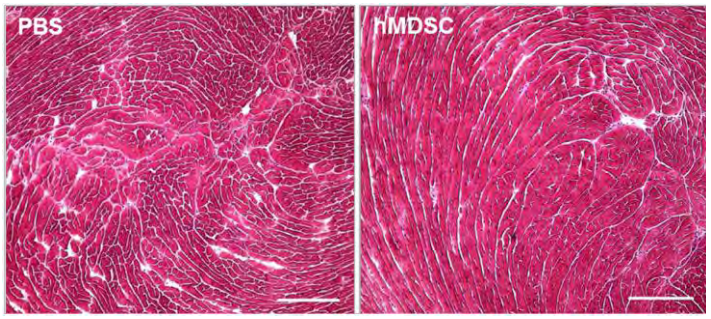
between the hMDSC and control groups, in all the test parameters, were observed immediately before the injection (all $p>0.05$). At 2 months post-injection, however, the LV contractile function had dramatically deteriorated in the control group (**Figure 6**). In sharp contrast, LV contractility was notably sustained following the hMDSC injection (LVFS, $p=0.006$; LVFAC, $p\leqq0.001$; LVEF, $p\leqq0.001$). Significant enlargement in LV chamber dimension was also documented in the control mice (**Figure 6**) at 2 month post-injection, suggesting progressive LV dilatation and ultimately heart failure. In the hMDSC-injected group, markedly smaller LV chamber sizes were observed (LVEDA, $p=0.011$; LVESA, $p\leqq0.001$), indicating prevention of adverse cardiac remodeling. Overall, these data suggest that a single intraperitoneal injection of hMDSCs not only sustains cardiac contractility but also ameliorates LV dilatation, collectively preventing progressive heart failure.

7) Intraperitoneal administration of hMDSC ameliorates myocardial fibrosis

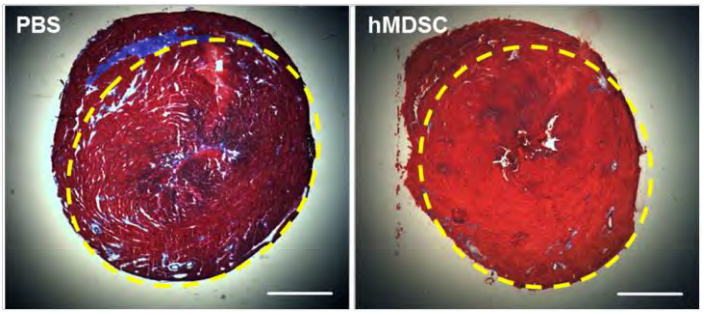
Using H&E and Masson's trichrome stainings, we investigated whether intraperitoneal hMDSC treatment could histologically reduce myocardial degeneration and fibrosis in the aging dystrophic heart. At 2-month post-injection, H&E staining revealed that the control group exhibited an extensive degenerative pattern throughout the left and right ventricular myocardium (**Figure 7A**). In contrast, the hMDSC-treated group showed less overall myocardial degeneration (**Figure 7A**). Masson's trichrome staining revealed that hMDSC-treated hearts displayed less interstitial collagen deposition (stained in blue) throughout the ventricular myocardium compared to the control hearts at 2-month post-implantation (**Figure 7B**). The estimation of the total fibrotic area ratio showed a significant 73.4% reduction of interstitial collagen deposition in the hMDSC-treated hearts (2.31±0.40%) when compared with the PBS-injected controls (8.68±1.94%) (**Figure 7C**, N=4 per group, $p=0.018$). These results suggest the prevention of progressive degeneration and interstitial fibrosis by hMDSC treatment.

Figure 7

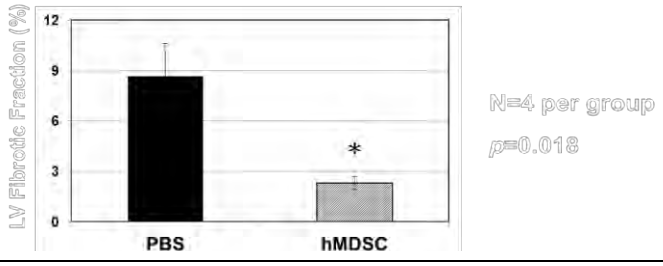
A



B

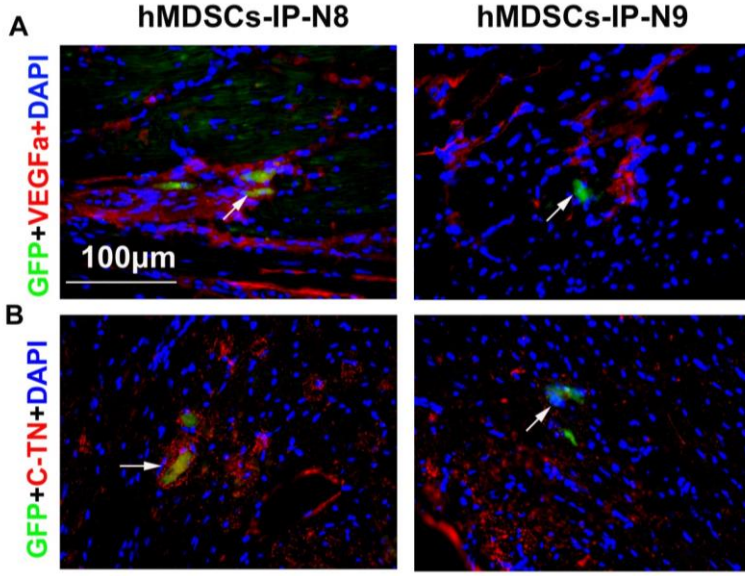


C



8) Immunoflurorescent staining of GFP and colocalization with VEGF and C-troponin

Figure 8



A: Very few GFP positive cells were found in the heart tissue. Some cells secrete VEGFa (N8, white arrow), some not (N9, white arrow). B. Dual color staining of GFP and cTN indicated GFP positive donor cells express cTN (N8, white arrow), some not (N9, white arrow).

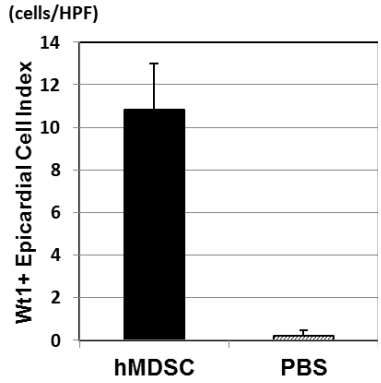
Heart sections from 2 different IP injected mice (N8 and N9) were dried at RT for 10 minutes and fixed with 4% PFA for 8 minutes. After 3 rinses with PBS, the sections were blocked with 5% donkey serum at RT for 1hr, and the primary antibodies (goat anti-GFP (ab6673, 1:200 dilution), rabbit anti-VEGFa (ab46154, 1:100), goat anti-C-troponin) were applied to the sections and incubated at 4^0C overnight. The slides were rinsed three times with PBS and donkey-anti-goat-488, donkey anti-rabbit-649, or donkey-anti-594 was applied to detect GFP in the green channel and VEGF in the infrared channel or C-troponin in the red channel. Secondary antibodies were incubated on the sections for two hours at room temperature. The sections were further washed with PBS three times and counter stained with DAPI for 10 minutes and rinsed with PBS and deionized water and mounted with aqueous medium. Fluorescent pictures were taken using a Nikon microscope utilizing Northern Eclipse software.

Our results indicated that there were only a very few donor cells engrafted in the heart tissues and only a few of those were VEGFa positive (**Figure 8A**). cTN and GFP colocalization indicated that there were also very few donor cells that expressed cTN (**Figure 8B**). This data supports our hypothesis that it is a paracrine effect that is primarily responsible for the beneficial effect the cells impart on the aged myocardium and not the cells direct differentiation into cardiomyocytes.

9) hMDSC treatment activates wt1+ epicardial progenitor cells

We further investigated whether the intraperitoneal injection of hMDSCs could activate resident wt1+ epicardial progenitor cells in the aging dystrophic heart. Almost no wt1+ cells were detected by immunohistochemistry in the epi/pericardium of the control hearts; however, wt1+ epicardial progenitor cells could be identified in clusters within the epi/pericardial layer of the hMDSC-injected mice (**Figure 9**, p=0.009)..

Figure 9



Progress made from: 10-1-12 to 9-30-13

1) ***Intraperitoneal injection of hMDSCs prevents progressive heart failure and promotes angiogenesis in the aging dystrophic hearts of mdx SCID mice.***

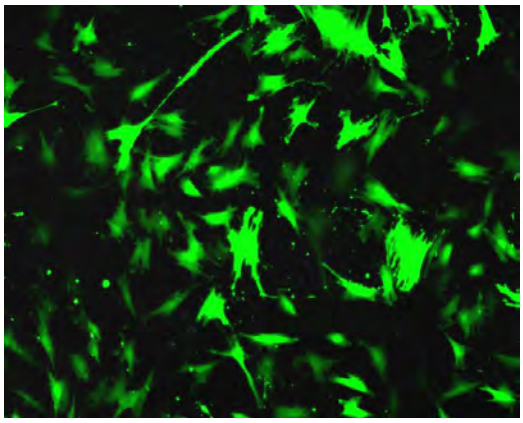


Figure 1: Sorted GFP+ hMDSCs

Cell isolation and labeling

Human muscle-derived stem cells (hMDSCs) were isolated using the modified pre-plate technique as previously described by our group (Okada, et al. Mol Ther 2012). hMDSCs were expanded in MDSC proliferation medium (MDSC-PM) containing high-glucose DMEM (Gibco), 10% fetal bovine serum (FBS, Invitrogen), 10% horse serum (HS, Invitrogen), 1% chicken embryo extract (CEE, Accurate Chemical), and 1% penicillin and streptomycin (P/S, Invitrogen). Cells were transduced with lentiviral vectors encoding GFP at MOI of 1:10 in the presence of 8μg/ml polybrene for 16hrs. After transduction, the cells were washed and expanded in MDSC-PM. Though a high transduction efficiency was achieved (~80%), the lentiviral-GFP transduced hMDSCs were further subjected to fluorescence-activated cell sorting (FACS) based on GFP signal positivity. The sorted GFP+ hMDSCs (>95% GFP positivity) were cultured and maintained in MDSC-PM following FACS (**Figure 1**).

Intraperitoneal injection and echocardiography in the dystrophic mouse model

To examine whether the intraperitoneal injection of hMDSCs could benefit the hearts of dystrophic mice, we used senile mdx/SCID mice (16-18 month old) that displayed symptoms of DMD. All mdx/SCID mice (total 10 mice) were randomly assigned to each group (N=5/group) before the start of the study. The experimental group received a single injection of 1×10^6 cells resuspended in 50ul of PBS in the lower abdomen while the control group received an equal amount of PBS in the same region. Cardiac function was assessed by echocardiography performed repeatedly immediately before (baseline) and after injection at 1 and 2 months. Animals that died prior to 2 month post-injection were excluded from the study. A total of 4 mice in the experimental group and 3 mice in the control group survived the 2-month experiment. Student's t-test was used for statistical analyses.

Intraperitoneal injection of hMDSCs prevented progressive heart failure

Left ventricular (LV) chamber size was measured by LV end-diastolic area (LVEDA, **Figure 2A**) and end-systolic area (LVESA, **Figure 2B**), whereas LV contractility was evaluated by LV fraction shortening (LVFS, Figure 2C), LV fractional area change (LVFAC, Figure 2D), and LV ejection fraction (LVEF, Figure 2E). Echocardiographic analyses showed notable enlargement in LV chamber dimension in the sham injected control group (Figure 2A-B), suggesting progressive LV dilatation and ultimately, heart failure. In the hMDSC-injected group, significantly smaller LV chamber sizes were observed at 2 month post-injection (LVEDA, p=0.024 and LVESA, p=0.008), indicating the prevention of progressive cardiac remodeling. Similarly, cardiac contractile function dramatically deteriorated in the control group over time (**Figure 2C-E**). Cardiac contractility was notably sustained following hMDSC injection for up to 2 months, including LVFS (p=0.054), LVFAC ($p \leqq 0.001$) and LVEF (p=0.004). Overall, these data suggest beneficial effects of intraperitoneal injection of MDSCs in aging dystrophic hearts, not only preventing progressive LV dilatation but also sustaining cardiac contractility.

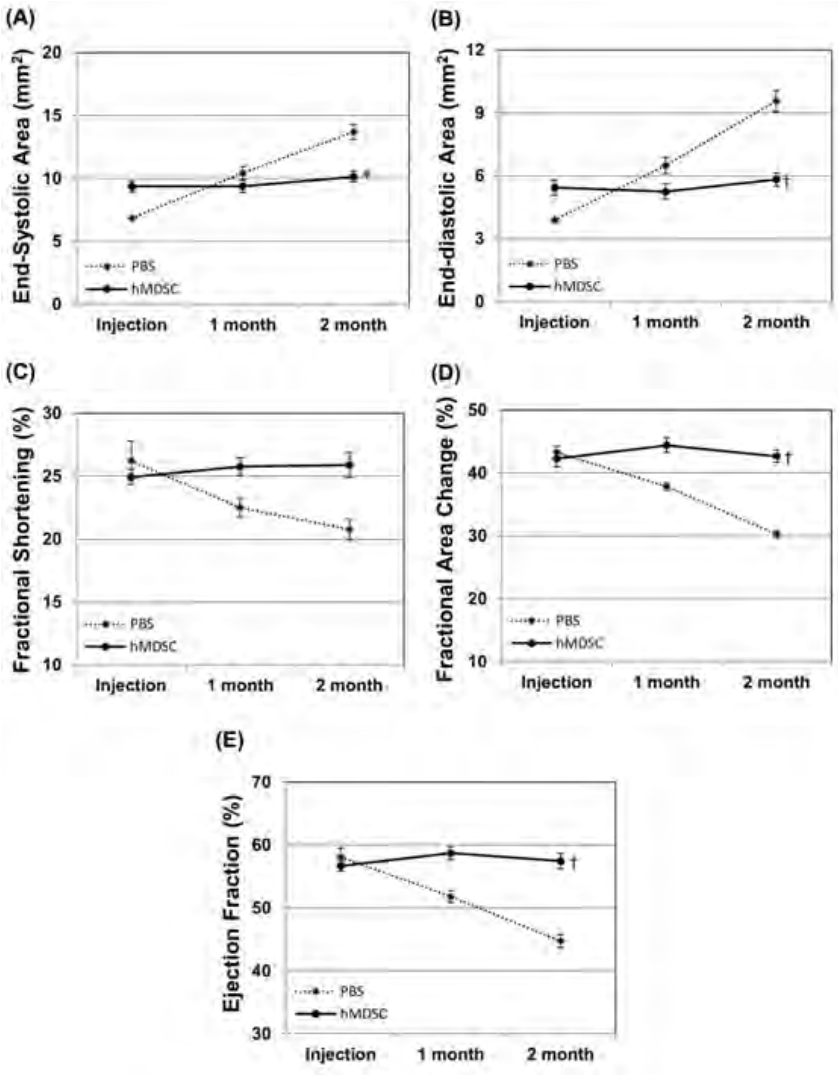


Figure 2. Echocardiographic analyses of LV chamber dimension and contractility

Intraperitoneal injection of hMDSCs promoted angiogenesis in the aging dystrophic heart of the mdx/SCID mice.

Immunohistochemical analysis revealed increased CD31+ capillary structures in the myocardium of hMDSC-injected mice (60.86±8.45/HPF) when compared with control mice (52.14±4.86/HPF) (Figure 3). Similarly, an increased number of alpha-smooth muscle actin (αSMA)-positive vessel structure was observed in hMDSC-injected mice (4.57±0.89/HPF) when compared with control mice (3.86±0.65/HPF) (Figure 4). Together these data suggest the promotion of angiogenesis and/or vasculogenesis in the myocardium following hMDSC intraperitoneal injection. Currently we hypothesize that this is due to the production of angiogenic and/or vasculogenic signaling molecules by the injected hMDSCs that subsequently travel to the heart via systemic circulation.

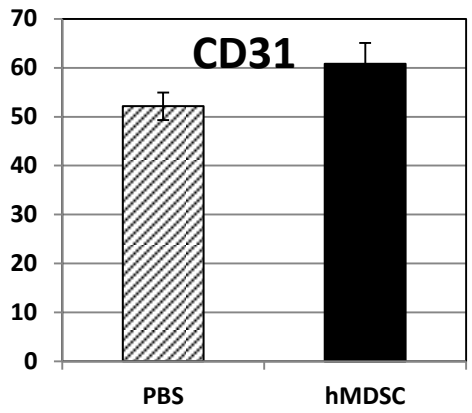


Figure 3. Immunohistochemical analysis of CD31+ capillary structures

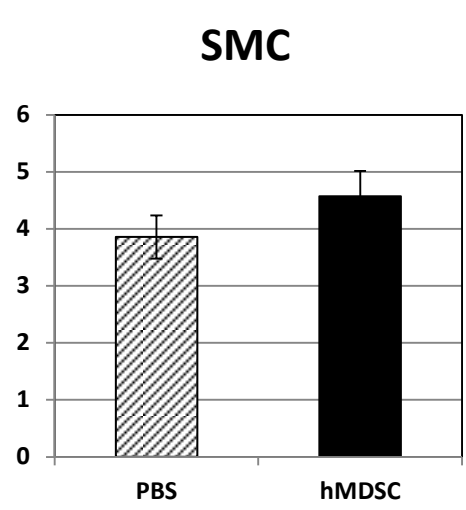
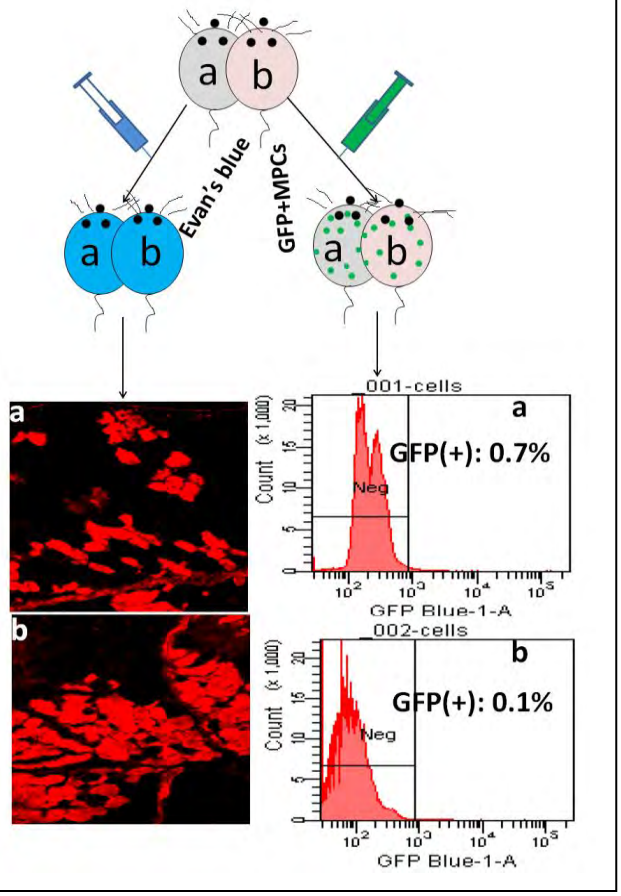


Figure 4. Immunohistochemical analysis of αSMA+ vessel structures

2) Potential benefit of parabiosis for cardiac repair in dKO mice

Based on the positive effect of RhoA inactivation in cardiac muscle of dKO mice, we proposed that the parabiotic pairing of dKO mice with normal mice could help repress the activation of RhoA signaling in the cardiac muscles of dKO mice, and therefore improve their defective muscle phenotype.

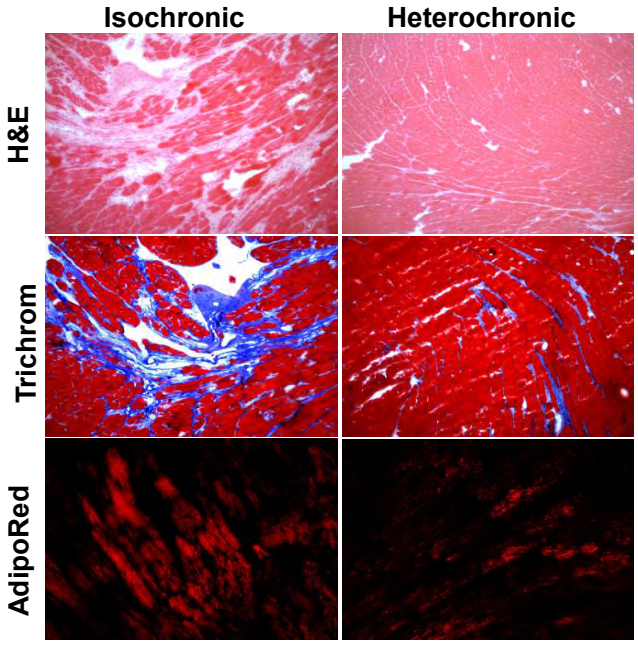
Figure 5



As a proof of concept and to verify the development of cross-circulation between the animals, we examined the distribution of an intravascular dye (Evan's Blue) across the joining wounds (**Figure 5**). After injection into one animal of the pair, the Evan's Blue dye immediately flushed the same animal **(a)** and progressively spread through the common vascular tree at the junction to its partner **(b)**. We also found that MPCs could travel from mouse **(a)** to the parabiotic partner **(b)** when GFP positive MPCs are injected intravenously (**Figure 5**).

Next we preformed heterochronic parabiotic pairings between old dKO-hetero mice and young mdx mice and tested whether the cardiac muscle histopathology could be improved by blood-borne factors in vivo which created a constant exchange of peripheral blood between the mice. We performed parabiosis between young mdx mice (3-5 months) and old dKO- hetero mice (12-14 months)**.** These heterochronic pairs were compared to the control isochronic pairs of two old dKO-hetero mice (12-14 months). H&E was performed to determine whether the cardiac muscles were more greatly improved in their histopathological appearance in the old dKO-hetero mice sutured with young mdx mice. Trichrome staining was also performed according to the manufacturer's instructions to determine whether less fibrosis formed in the old mice sutured with young mice. Intramyocardial lipid accumulation, which is a precursor of myocardial degradation and can cause myocardial toxicity and heart failure, was also determined via AdipoRed staining. We found that there was decreased fibrosis and an improvement in the histology of the cardiac muscle, and a decrease in intramyocardial lipid accumulation in the cardiac muscle of old dKO-hetero mice after they were sutured together with young mdx mice for 3 months (**Figure 6**). The results suggest that the defect in the MPCs might be related to the dystrophic microenvironment or some circulating factors. These observations suggest that changes in the dystrophic microenvironment could be a new approach to improve cardiac muscle weakness in DMD patients, despite the continued lack of dystrophin expression.

Figure 6



3) Myocardial Calcification and Fibrosis in Dystrophic Mice is Reduced by RhoA Inactivation

Myocardial calcification refers to the excessive deposition of calcium in the cardiac muscle and is usually observed in the aging population as-well-as long-term survivors of substantial myocardial infarctions (1-2). Myocardial calcification could either be the result of chronic degeneration or reflect ongoing pathologic processes (2); h*owever, the cellular and molecular mechanisms leading to* myocardial calcification *remains largely unknown.* We have recently reported the observation of extensive skeletal muscle calcification/heterotopic ossification in the dystrophin/utrophin double knockout (dKO) mouse model of Duchenne muscular dystrophy (3), which could be mediated by the over-activation of the RhoA signaling in muscle stem cells (MSCs) (4). RhoA is a small GTPase protein that regulates cell morphology and migration in response to extracellular signaling and stresses. RhoA activation in MSCs induces their osteogenesis potential, inhibits their adipogenic potential, mediates BMP-induced signaling, and promotes osteoblastic cell survival (5). The involvement of RhoA in mediating inflammatory processes and myocardial fibrosis has previously been described (6). In addition, previous studies have indicated that the sustained activation of the RhoA pathway can block the differentiation of muscle cells by inhibiting myoblast fusion (7). Cardiac involvement is the leading cause of early death in DMD patients, and the current study was performed to elucidate the role of RhoA in mediating fibrosis and calcification in the cardiac muscle of dystrophic mice.

Methods:

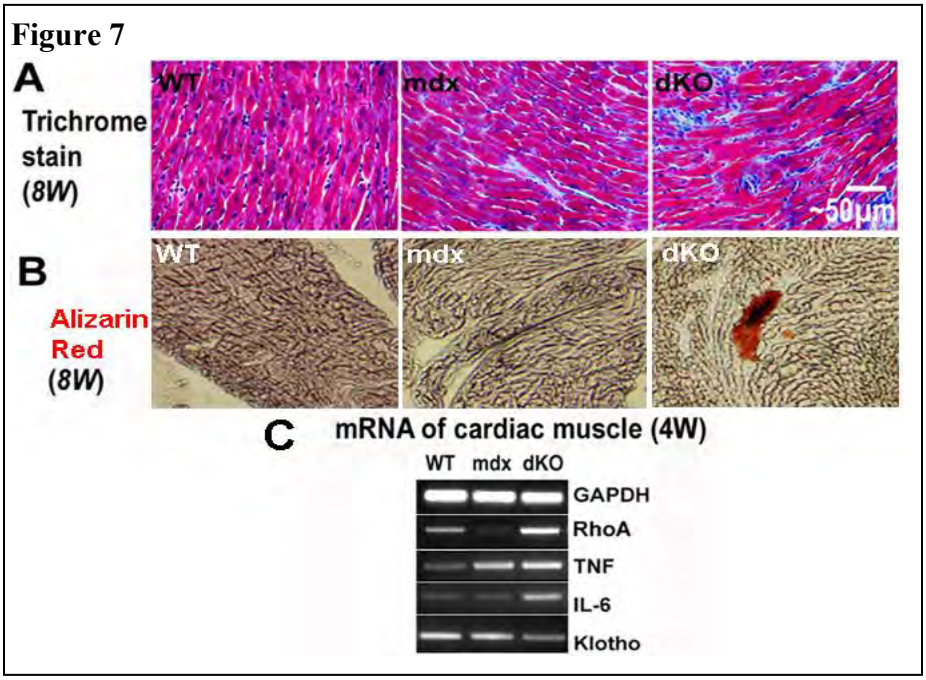
1. Animals: WT, mdx and dKO mice were used in this experiment **2. RhoA inactivation with Y-27632:** dKO mice from 3 weeks of age received either an intraperitoneal (IP) injection of Y-27632 [5mM in Phosphate Buffered Saline (PBS), 10mg/kg per mouse], which is a systematic inhibitor of RhoA signaling or PBS only (control). IP injections were conducted 3 times a week for 4 weeks. **3. Histology:** Mice were sacrificed and 10µm cryostat sections were prepared from the cardiac muscles of the mice. Alizarin red stain was conducted to stain calcium deposition in the cardiac muscle. **4. Statistics**: N >=6 for each group. Student's T-test was used to evaluate for significance.

Results:

a) ***Cardiac muscle of dKO mice featured increased fibrosis and calcification when compared to mdx and WT mice.***

Trichrome staining of the cardiac muscles from 8-week old WT, mdx, and dKO mice was conducted to characterize ECM collagen deposition, which revealed that fibrosis formation was generally severe in the dKO mice, mild in the mdx mice, and absent in WT mice (**Figure 7A**). Alizarin red staining of the cardiac muscle revealed that calcification occurred in the dKO mice (**Figure 7B**), but not in the WT or mdx mice.

Figure 7



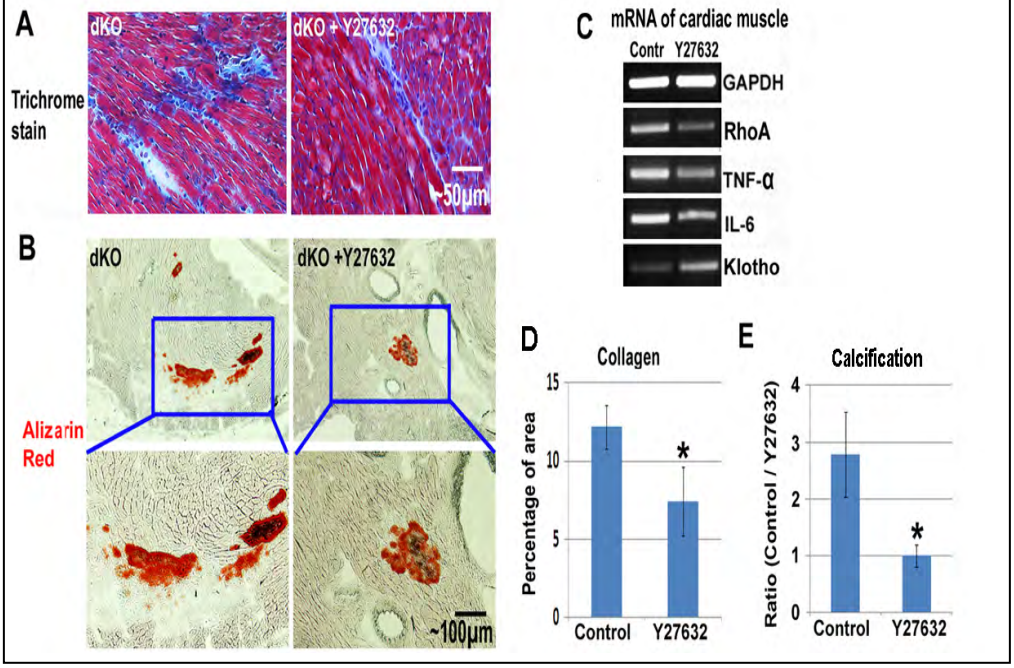
b) Up-regulation of RhoA in the cardiac muscle of the dKO mice.

Semi-quantitative PCR showed that, compared to the WT and mdx mice, the expression of RhoA and the inflammation signaling genes, TNF-α and IL-6, was up-regulated in the dKO cardiac muscle, while the expression of the anti-inflammation gene Klotho was down-regulated (**Figure 7C**). We suggest that the activation of RhoA and inflammatory mediators are involved in the cardiac fibrosis and calcification seen in dKO mice.

c) Systemic RhoA inactivation via intraperitoneal injection (IP) of Y-27632 reduced fibrosis and HO in dKO cardiac muscle.

We hypothesized that RhoA inactivation could reduce fibrosis and calcification in the cardiac muscles of the dKO mice. To confirm this hypothesis, Y-27632 was injected intraperitoneally (IP) to achieve the systemic inhibition of RhoA signaling in 3-week old dKO mice. As expected, after 4 weeks of continuous injection. Semi-quantitative PCR revealed that the expression of RhoA, TNF-α and IL-6 was down-regulated with Y-27632 administration, while the expression of Klotho was up-regulated (**Figure 8E**). Fibrosis and calcification in the cardiac muscles of dKO mice was decreased compared to non-treated mice (**Figure 8A-D**).

Figure 8



Discussion:

Our data reveals the involvement of RhoA signaling in regulating calcification and fibrosis of cardiac muscle, and indicates RhoA may serve as a potential target for repressing injury-induced and congenital cardiac muscle calcification and fibrosis in humans.

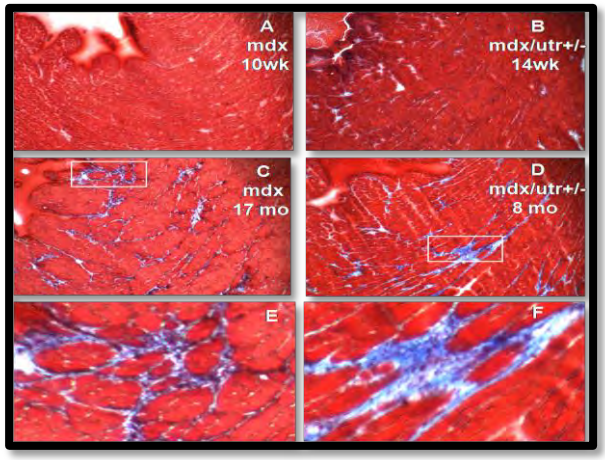
Both calcification and fibrosis in cardiac muscle is typically the result of a pathologic process. Our current results revealed that the RhoA signaling pathway may mediate the calcification and fibrosis processes in the cardiac muscles of dKO mice. RhoA seems to be co-activated with inflammatory signaling in severely dystrophic cardiac muscle, and the inactivation of RhoA signaling could repress this signaling. Therefore, our results indicate that the involvement of RhoA signaling in the therapeutic prevention of calcification and fibrosis in cardiac muscle should be further investigated as a potential target for treating DMD patients and other pathologic conditions of the heart.

Progress made from: 10-1-11 to 9-30-12

1) Double Utrophin/dystrophin knockout mice (dKO mice) develop a more severe cardiomyopathy than the mdx mice.

DMD patients die of cardiac or respiratory failure in their 3rd decade (8). The *Mdx* mouse model has near-normal cardiac function until very late in life; therefore, we aimed to determine if dystrophin-/- utrophin+/- (double knock-out, DKO het) mice might represent a superior model of DMD associated cardiomyopathy, and test it to see if we could eventually treat DMD associated heart failure via cell therapy. Some hallmarks of cardiomyopathy include inflammation and fibrosis which leads to decreased contractility and thus dysfunction of the heart. Left ventricle dilation and wall thinning which leads to a decreased ability to pump blood

Figure 1



(contractile dysfunction), decreased angiogenesis, which leads to greater cell death due to a of lack of appropriate nutrients and oxygen , and arrhythmias (9). First we assayed and compared the amount of fibrosis in the hearts of mdx and DKO het mice at the ages of 10 weeks to 17 months. We found similar levels of fibrosis in mdx mice at 10 weeks and DKO het mice at 14 weeks (**Figure 1A and B**); however, the DKO het mice appeared to accumulate cardiac fibrosis more rapidly than mdx mice. The DKO het mice at 8 months had similar fibrosis levels to mdx mice at 17 months (**Figure 1C -F**).

Therefore we went on to further characterize the hallmarks of cardiomyopathy in DKO het mice aged 6-10 months. First we looked at global fibrosis from 6-10 months and found rapidly increasing fibrosis levels (**Figure 2**). Next, we characterized the cardiac function looking at end diastolic area and fractional area change. Dilation of the hearts increased with the age of the mice which is shown in **Figure 3** and quantified in **Figure 4** as end diastolic area. Correspondingly, the cardiac functional parameter fractional area change decreases from 6-10 months (**Figure 4**).

Figure 2

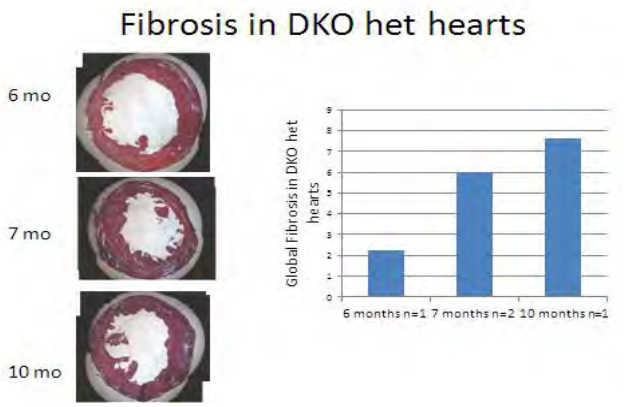


Figure 3

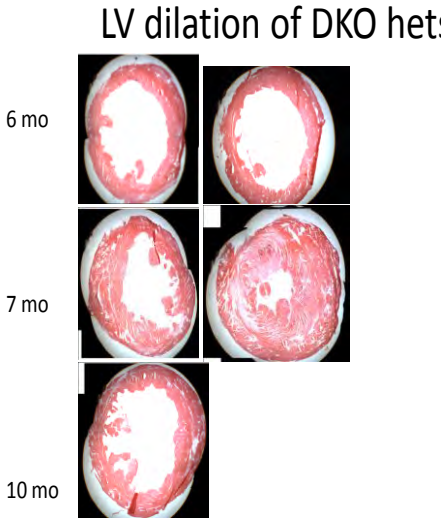
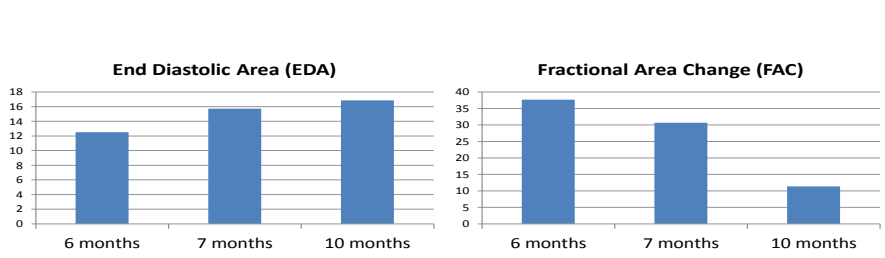
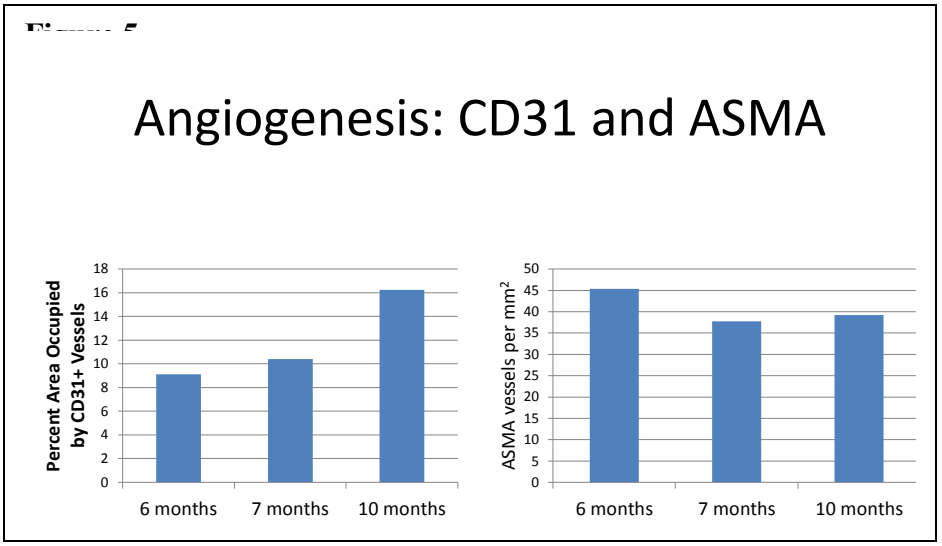


Figure 4



Finally, we examined angiogenesis in the DKO het hearts and found that the percent area of CD31+ endothelial cells increased at 10 months; however, the number of alpha smooth muscle actin positive cells, which marks more mature vascular structures, decreased from 6-7 months and then remained fairly constant through to 10 months (**Figure 5).**



In conclusion, as DKO mice age cardiac fibrosis levels, dilation, and FAC get progressively worse making them a better model for DMD associated cardiomyopathy than the mdx mice, which do not develop severe cardiomyopathy until much later in life. Therefore, we have selected to use the DKO mouse model for the remaining of the experimental objectives which aim to develop biological approaches based on muscle derived stem cells to improve cardiac function after cardiomyopathy.

2) ***Intramyocellular lipid accumulation, fibrosis and HO in cardiac muscle of dKO mice are more extensive then in mdx mice: The role of RhoA in the cardiac histopathology observed in dKO mice.***

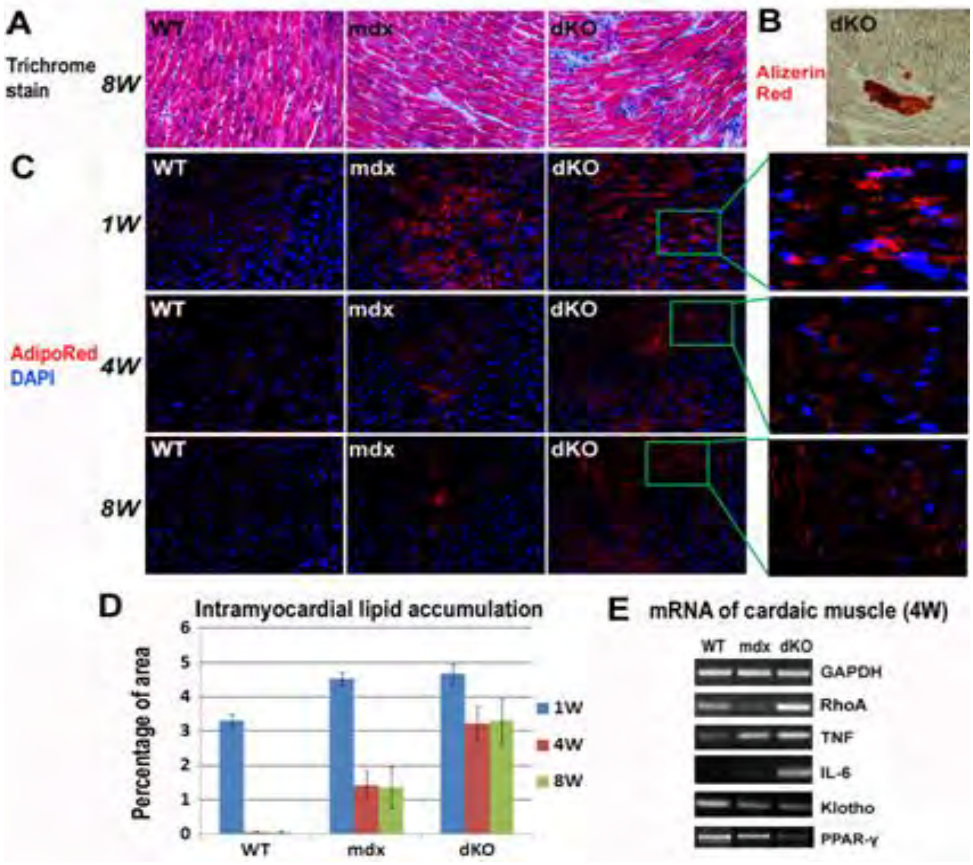
Heterotopic ossification (HO) or ectopic fatty infiltration often occurs in muscle tissues in various disease states, and can contribute to impaired metabolism, muscle force production, and mobility function (10-11). HO is a process where bone tissue forms in soft tissues, which can be caused by trauma, surgery, neurologic injury and genetic abnormalities (11). Bone Morphogenetic Proteins (BMPs) are known to be responsible for the induction of HO in damaged skeletal muscle (12). Previous studies in our laboratory demonstrated that HO induced by BMP-4, demineralized bone matrix, and trauma in skeletal muscle mouse model could be repressed by overexpressing Noggin, a BMP antagonist (13). Fatty infiltration is the accumulation of fat cells outside the typical fat stores underneath skin, and has been reported to be associated with aging, inactivity, obesity, and diseases like diabetes (10, 14-15). Fatty infiltration into skeletal muscle (intramuscular adipose tissue, IMAT) is often associated with disorders in lipid metabolism (14-16); however, disorders in lipid metabolism in skeletal muscle can often cause another type of abnormal lipid deposition: intramyocellular lipid accumulation (17-19). More important for the current proposal, intramyocellular lipid accumulation also occurs in cardiac muscle (intramyocardial lipid accumulation) which can also be similarly caused by disorders in lipid metabolism or lipid overload, which leads to lipotoxicity and cardiac dysfunction, and can be a sign of myocardial degradation during the progression of cardiac dysfunction *(20-24).* Intramyocellular lipid accumulation in cardiac muscle (intramyocardial lipid accumulation) has been observed in DMD patients, especially in the most damaged area of the heart (25-26). Here we suggest that intramyocellular lipid accumulation may occur in the cardiac muscles of DKO mice as well and could be correlated with the progress of phenotypes such as fibrosis formation.

RhoA is a small G-protein in the Rho family that regulates cell morphology and migration in response to extracellular signals, via reorganizing actin cytoskeleton (27-28). The RhoA-Rho kinase (ROCK) signaling pathway functions as a commitment switch for osteogenic and adipogenic differentiation of mesenchymal stem cells (MSCs) (5). Activation of RhoA-ROCK signaling in cultured MSCs *in vitro* induces their osteogenesis but

inhibits the potential of adipogenesis, while the application of Y-27632, a specific inhibitor of ROCK, reversed the process (5, 29-30). RhoA-ROCK inhibitor Y-27632 was found to induce adipogenic differentiation of myofiber-derived muscle cells *in vitro,* and resulted in fatty infiltration in skeletal muscle (31). Meanwhile, RhoA was shown to be activated by Wnt5a in inducing osteogenic differentiation and repressing adipogenic differentiation of human Adipose Stem Cells (ASCs) (32). Furthermore, the role of RhoA signaling in inflammatory reactions has been demonstrated, for example, TNF-α induces activation of RhoA signaling in smooth muscle cells (33), and RhoA was found to regulate Cox-2 activity in fibroblasts (34). In addition, an important role of RhoA in myogenic differentiation has also been demonstrated where the sustained activation of the RhoA pathway can block muscle differentiation by inhibiting myoblast fusion (7, 35-36).

Trichrome staining of cardiac muscles from WT, mdx and dKO mice (8-weeks old) was conducted to characterize extracellular matrix (ECM) collagen deposition in the muscles. Our findings showed that fibrosis formation was generally very severe in dKO mice, mild in mdx mice, and absent in WT mice (**Figure 6A**). Alizarin Red staining of the cardiac muscle also revealed the occurrence of HO in the cardiac muscle of dKO mice (**Figure 6B**), but not in WT and mdx mice (data not shown). Meanwhile, we did not observe obvious fatty infiltration in the cardiac muscle of any of the three mouse models (data not shown). Intramyocardial lipid accumulation was observed at 1 week of age in all 3 mouse models (**Figure 6C**); however, intramyocardial lipid accumulation in fetal WT mice is known to be common, because unlike the adult hearts, the fetal heart uses glucose and not fatty acids as sources of energy (37-38). Intramyocardial lipid accumulation was found to decrease rapidly in WT mice from one week after birth, and became nearly non-detectable by 4 weeks of age (**Figure 6C**); however, both the mdx and dKO mouse models still exhibited extensive amounts of intramyocardial lipid (**Figure 6C, D**), which was far more pronounced in the DKO mice than the mdx mice (**Figure 6C, D**). Compared to WT and mdx mice, the expression of RhoA and inflammatory signaling genes (TNF-α and IL-6) was found to be more up-regulated in the cardiac muscle of dKO mice, while the expression of Klotho gene was down-regulated (**Figure 6E**). We suggest that the activation of RhoA and inflammatory signaling may account for the higher levels of HO, intramyocardial lipid accumulation and fibrosis observed in the DKO cardiac muscles.

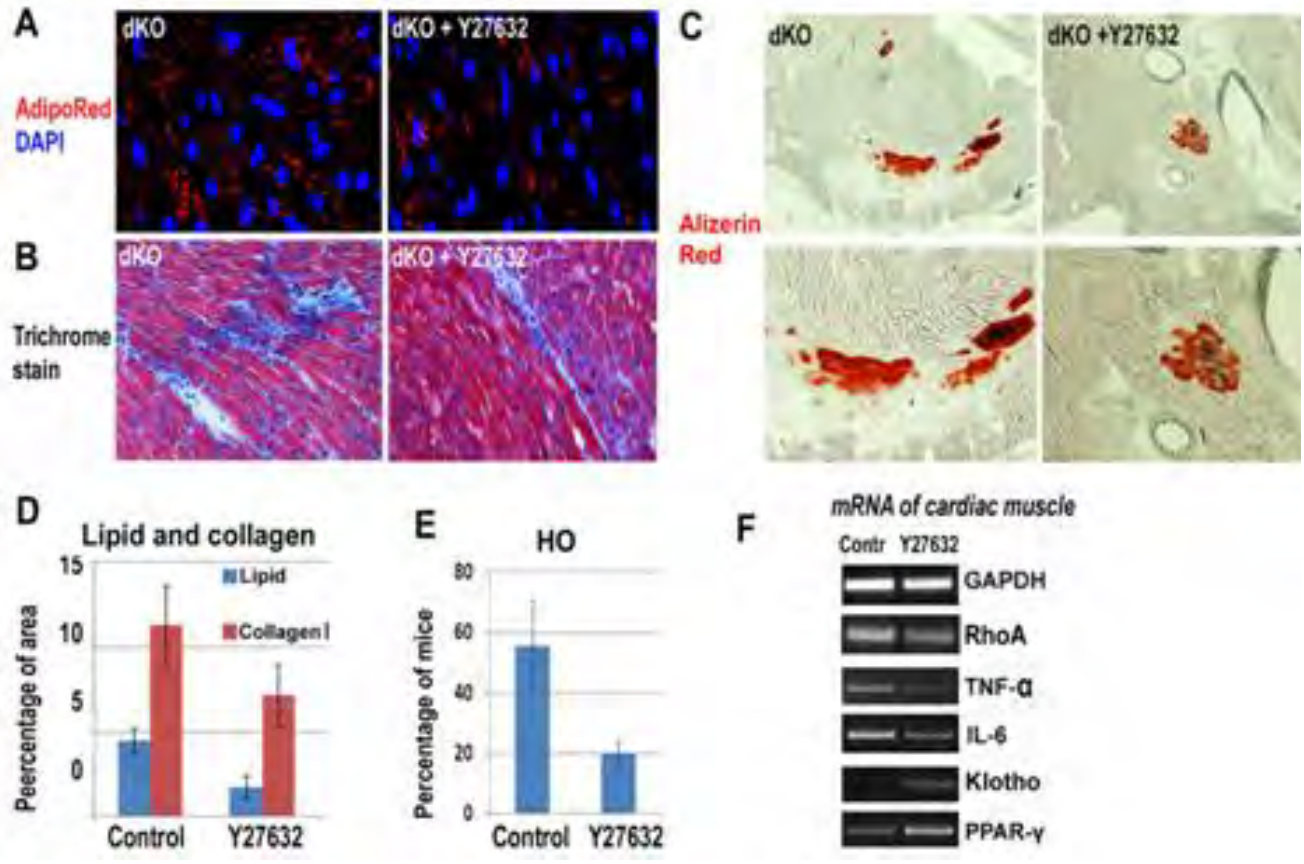
Figure 6



In vivo RhoA inactivation in DKO mice reduces intramyocellular lipid accumulation fibrosis, and HO in cardiac muscle.

We hypothesized that RhoA inactivation could reduce HO, intramyocellular lipid accumulation and fibrosis in the cardiac muscles of DKO mice. To verify this hypothesis, Y-27632 was injected intraperitoneally (IP) to achieve systematic inhibition of RhoA signaling in DKO mice (4-week old). After 3 weeks of continuous IP injection, the skeletal muscle phenotype of the DKO mice was found to be similarly improved as when we performed local injection of Y-27632 into skeletal muscle (data not shown). As anticipated the intramyocellular lipid accumulation, fibrosis and HO in cardiac muscle was also reduced (**Figure 7A-E**). Semi-quantitative PCR studies on the cardiac muscle tissues further revealed that, the expression of RhoA and inflammatory factors was down-regulated with Y-27632 administration and the expression of Klotho and PPARγ was up-regulated (**Figure 7F)**.

Figure 7



3) The identification of human muscle progenitor cells for dystrophic heart repair and regeneration.

<u>Human myo-endothelial cells repair injured cardiac tissue effectively</u>

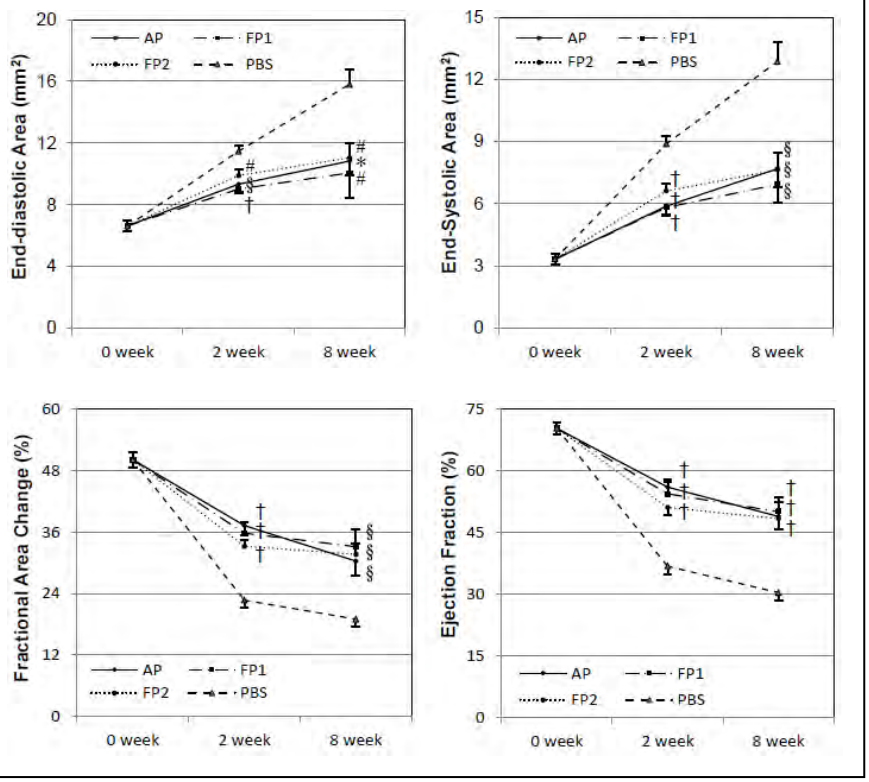
The potential of human myo-endothelial cells (MECs, CD34+/144+/56+/45-) to repair the injured heart was demonstrated in our recent study. When transplanted intramyocardially into acutely infarcted immunodeficient hearts of young adult mice, MECs more effectively restored cardiac function, reduced scar tissue formation, and

stimulated angiogenesis than purified conventional myoblasts and endothelial cells (39). This is presumably attributed to, at least in part, the augmented angiogenesis, resulted from more vascular endothelial growth factor (VEGF), a potent angiogenic factor, secreted by MECs under hypoxia. Similar to murine MDSCs, MECs regenerated significantly more fast-skeletal MHC-positive myofibers in the ischemic heart. A small fraction of engrafted MECs expressed cardiomyocyte markers, cardiac troponin-T and –I, indicating the likely cardiac differentiation and/or cell fusion in the injured heart. MECs also promoted proliferation and prevented apoptosis of endogenous cardiomyocytes. These results suggest that human MECs are an ideal donor cell population for cardiac repair.

Human pericytes restored the function of the injured heart through paracrine effect and cellular interaction

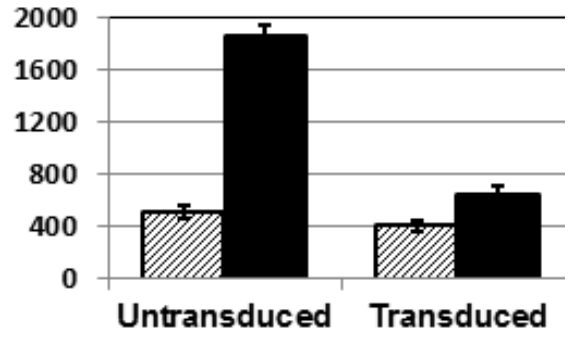
Human microvascular pericytes ($CD146^{+}/34^{-}/45^{-}/56^{-}$), with their inherent vascular functions and recently documented multipotency, matched the scope of ideal cells for cardiac repair. (40). Transplantation of cultured human muscle pericytes into acutely infarcted hearts of immunodeficient mice significantly improved cardiac contractility and reduced heart dilatation, superior to CD56+ myogenic progenitor transplantation (**Figure 8**). Pericytes exhibited cardio-protective effects such as promotion of angiogenesis, decrease of chronic inflammation, and reduction of scar formation (41). Under hypoxia, pericytes suppressed murine fibroblast proliferation and macrophage proliferation *in vitro* through a paracrine mechanism. Pericytes demonstrated expression of a number of immunoregulatory molecules, including IL-6, LIF, COX-2 and HMOX-1, even under hypoxia. Pericytes not only supported microvascular structure formation in three-dimensional co-cultures but also significantly enhanced host angiogenesis *in vivo*. Under hypoxia, pericytes dramatically increased expression of VEGF-A, PDGF-β, TGF-β1.

Figure 8



In conclusion, intramyocardial transplantation of purified human muscle pericytes promotes functional and structural recovery, attributable to multiple mechanisms involving paracrine effects and cellular interactions.

Figure 9



Currently, to examine whether donor pericyte-derived VEGF is the primary molecular mediator required for the promotion of host angiogenesis, we used anti-human $VEGF_{165}$ shRNA to block VEGF secretion from donor pericytes. The angiogenic effect of transduced pericytes with VEGF blockage as well as their overall therapeutic benefits post-transplantation will be assessed. Human muscle pericytes were successfully transduced by lentiviral anti-VEGF-shRNA-eGFP vector. The transduction rate was roughly 70-80%. We observed no significant change to cell growth and phenotypes after transduction. To further purify transduced pericytes, we plan to use FACS to sort GFP-positive pericytes to homogeny. *In vitro* hypoxia assay showed that although anti-VEGF-shRNA transduction only reduced VEGF secretion from pericytes by 22.3% under normoxia, it effectively inhibited their VEGF secretion by 65.2% under hypoxia, as revealed by ELISA (**Figure 9**). To examine whether their vessel-forming capacity is affected by VEGF blockage, the Matrigel culture assay using pericytes has been performed and the data is currently being analyzed.

Figure 10

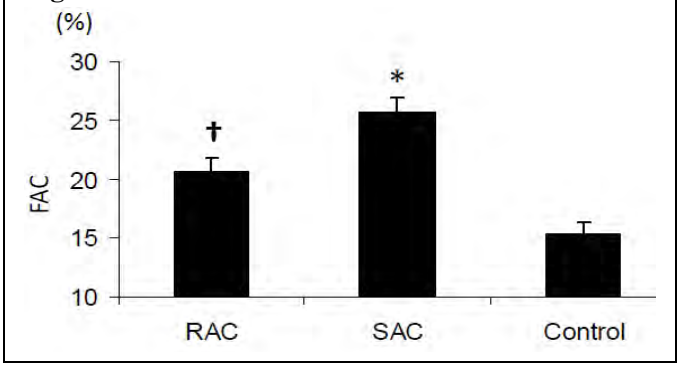


Figure 11

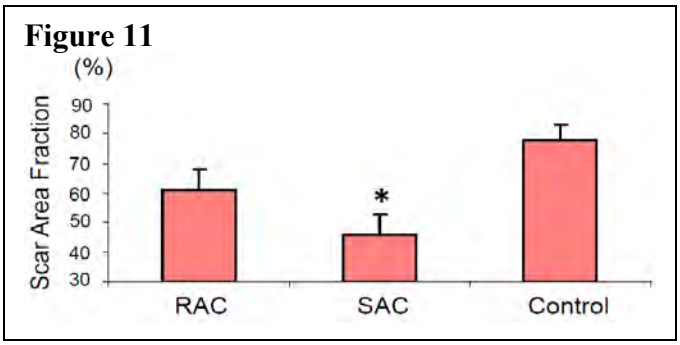
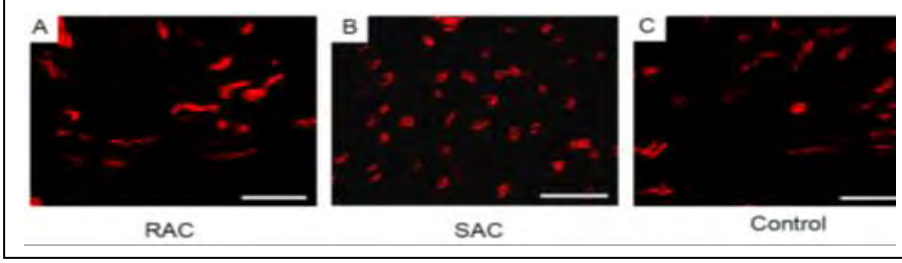


Figure 12



<u>Pre-plated human muscle cells display a differential cardiac repair capacity.</u>

Very recently, we applied the modified pre-plate technique to separate freshly dissociated human muscle cells into rapid- and slowly-adhering cell populations, equivalent to the murine early and late pre-plating cells. The slowly-adhering cells (SACs) displayed not only a greater myogenic potential but also better cell survival under oxidative and inflammatory stresses *in vitro* than rapidly-adhering cells (RACs), similar to murine MDSCs (Okada, Payne et al., Mol. Ther. 2012). We have investigated the therapeutic potential of human muscle pre-plating cells for the treatment of myocardial injury. The intramyocardial injection of SACs into the acutely infracted immunodeficient murine heart improved cardiac contractility more effectively than the injection of RACs (**Figure 10**; (42)). The functional recovery likely resulted from the decreased myocardial fibrosis (**Figure 11**), higher cell proliferation, reduction of cardiomyocyte apoptosis and increased revascularization (**Figure 12**) in SAC-injected hearts, similar to the therapeutic capacity of murine MDSCs.

Technical Issues

As indicated previously our mdx/SCID model does not exhibit prominent loss of cardiac function and dystrophic phenotypes in the cardiac tissue until they are nearly 2 years of age. We therefore have continued to age our mdx/SCID mice in order to obtain an aged colony of mice that exhibit prominent cardiomyopathy. We have begun full-scale experiments utilizing mice at the ages of 16-18 months, and the results outlined above have been extremely encouraging. We will continue to age our colony and perform additional experiments with this model over the up-coming year with multiple populations of human muscle-derived cells.

Future Direction

1) **Continue to age our mdx/SCID colony and perform additional full-scale IP experiments to compare the effect different populations of human MDCs have on improving the cardiomyopathy in the mdx/SCID mice.**
2) **We will also investigate the role that angiogenesis plays in the regeneration/repair capacity of human MDSCs transduced with VEGF or sFLT-1 and injected IP.**
3) **Parabiosis experiments will be performed to try and identify the potential protective factors that are shared by young unaffected mice when parabiotically paired with old dKO or old mdx/SCID animals that are presenting with cardiomyopathy.**

KEY RESEARCH ACCOMPLISHMENTS:

- **Identified and characterized a superior model of dystrophic related cardiomyopathy (the dKO mouse).**
- **Identified the relationship between the activation of RhoA and inflammatory signaling which appears to account for the higher levels of heterotopic ossification (HO), intramyocardial lipid accumulation and fibrosis observed in the dKO cardiac muscles.**
- **Demonstrated that in vivo RhoA inactivation with Y-27632 in dKO mice reduces intramyocellular lipid accumulation fibrosis, and HO in cardiac muscle.**
- **Demonstrated that purified human pericytes could restore the function of the injured heart through paracrine effects and cellular interaction**
- **Demonstrated that the intramyocardial injection of preplate isolated SACs into the acutely infracted immunodeficient murine heart improved cardiac contractility more effectively than the injection of RACs.**
- **Demonstrated that the intraperitoneal injection of human MDSCs could prevent progressive heart failure and promote angiogenesis in the dystrophic hearts of aged mdx SCID mice.**
- **Demonstrated that the parabiotic pairing of a young unaffected mouse with a dystrophic mouse could reduce cardiac fibrosis and intramyocardial lipid accumulation in the old dystrophic mice, which could lead to the identification of circulating factors from the young unaffected animals that could improve cardiac muscle weakness and cardiomyopathy in dystrophic mice without the need to replace dystrophin. This could lead to potential new therapies to treat DMD patients.**
- **Demonstrated that RhoA signaling regulates calcification and fibrosis of cardiac muscle, and indicates RhoA may serve as a potential target for repressing injury-induced and congenital cardiac muscle calcification and fibrosis in human patients.**

- **Demonstrated that under hypoxic culture conditions (oxidative stress), vascular endothelial cell growth factor (VEGFa) expression was significantly upregulated in the hMDSCs, while insulin like-growth factor-1(IGF-1), hepatocyte growth factor (HGF), and angiopoietin 2 (ANG2) were all down-regulated significantly.**
- **Demonstrated that TNFα treatment (Inflammatory stress) significantly increased the expression of IGF1 and IGF2, but down regulated HGF, angiopoietin 1 and angiotensinogen (AGT) expression by the hMDSCs.**
- **Compared RNA expression levels of 12 different population of cells including: 4 populations of human MDSCs isolated from different donors (23yo male, 20yo male, 31yo female, 76yo female), 4 populations of human PP1 fibroblasts isolated from the same donors, human heart pericytes, human myoblasts, human cardiac fibroblasts, and human skeletal muscle pericytes.**
- **Performed full scale IP injection experiments on aged mdx/SCID mice exhibiting cardiomyopathy comparing hMDSCs, Myoblasts and PBS and found:**
    - ***Intraperitoneal administration of human muscle-derived stem cells prevent progressive failure of the aging dystrophic heart.***
    - ***Intraperitoneal administration of hMDSC ameliorates myocardial fibrosis.***
    - ***That a paracrine effect is primarily responsible for the beneficial effect the IP injected cells impart on the aged myocardium and not the cells direct differentiation into cardiomyocytes.***
    - ***hMDSC treatment activates wt1+ epicardial progenitor cells.***

REPORTABLE OUTCOMES:

1. **RhoA signaling regulates heterotopic ossification and fatty infiltration in dystrophic skeletal muscle.** Xiaodong Mu, Arvydas Usas, Ying Tang, Aiping Lu, Jihee Sohn, Bing Wang, Kurt Weiss, and Johnny Huard. Orthopaedic Research Society; 2013 ORS Annual Meeting; January 26-29, 2013; San Antonio, TX. (**Appendix 1**)
2. **RhoA inactivation represses BMP-induced heterotopic ossification (HO) in skeletal muscle and potential for HO treatment.** Xiaodong Mu, Kurt Weiss, and Johnny Huard. Orthopaedic Research Society; 2013 ORS Annual Meeting; January 26-29, 2013; San Antonio, TX. (**Appendix 2**)
1. **Human Pericytes for Ischemic Heart Repair.** Chien-Wen Chen, Masaho Okada, Jonathan D. Proto, Xueqin Gao, Naosumi Sekiya, Sarah A. Beckman, Mirko Corselli, Mihaela Crisan, Arman Saparov, Kimimasa Tobita, Bruno Péault, Johnny Huard. Stem Cells, 2013 Feb; 31(2):305-16. Doi:10.1002/stem.1285. PMID: 23165704. (**Appendix 3**)
3. **Human skeletal muscle cells with a slow adhesion rate after isolation and enhanced stress resistance improve functions if ischemic heart.** Okada M, Payne T, Drowley L, Jankowski R, Momoi N, Beckman S, Chen C, Keller B, Tobita K, Huard J. Mol Ther 2012 Jan; 20(1):138-45. PMID: 22068427 (**Appendix 4)**
4. **Myocardial Calcification and Fibrosis in Dystrophic Mice is Reduced by RhoA Inactivation.** Xiaodong Mu, Ying Tang, Koji Takayama, Bing Wang, Weiss Kurt, and Johnny Huard. Orthopaedic Research Society, 2014 Annual Meeting, March 15-18, New Orleans, LA. (**Appendix 5**)
5. **The Role of Antioxidation and Immunomodulation in Postnatal Multipotent Stem Cell-Mediated Cardiac Repair.** Saparov A, Chen C-W, Beckman SA, Wang Y, Huard J. International Journal of Molecular Sciences. 2013; 14(8):16258-16279. **(Appendix 6**)
6. **RhoA mediates defective stem cell function and heterotopic ossification in dystrophic muscle of mice,** Xiaodong M, Usas A, Tang Y, Lu A, Wang B, Weiss K, Huard J. FASEB Journal. 2013 May 23. PMID: 23704088. (**Appendix 7**)

7. **Intraperitoneal administration of human muscle-derived stem cells reduce interstitial fibrosis, activate Wt1+ epicardial progenitor cells, and prevent cardiac dysfunction in the aging dystrophic heart.** William C.W. Chen, Talgat Yessenov, Aiping Lu, Xueqin Gao, Kimimasa Tobita, Arman Saparov, and Johnny Huard. ISSCR 2014 Annual Meeting, June 18-21, Vancouver, CAN. (**Appendix 8**)
8. **Human muscle-derived stem cells prevent cardiac dysfunction in the aging dystrophic heart.** William C.W. Chen, Xueqin Gao, Talgat Yessenov, Kimimasa Tobita, Arman Saparov, Aiping Lu, and Johnny Huard. ASCGT 2014 Annual Meeting, May 20-24, Washington, DC. (**Appendix 9**)

CONCLUSIONS:

Our current results during this period (10-1-13 to 9-30-14) confirmed our very preliminary findings from last year, utilizing an n=6 for each experimental groups (hMDSC, myoblasts and PBS), that IP injection of hMDSCs impart a significant beneficial effect on the aging dystrophic hearts of mdx/ SCID mice, not only preventing progressive LV dilatation but also sustaining cardiac contractility. Moreover, our recent data found that the intraperitoneal administration of hMDSCs ameliorated myocardial fibrosis and activated wt1+ epicardial progenitor cells. Our results showed that very few donor cells engrafted in the heart tissue and that only a few of those cells were VEGFa positive and cTN and GFP colocalization indicated that there were also very few donor cells that expressed cTN, which supports our hypothesis that it is a paracrine effect that is primarily responsible for the beneficial effect the cells impart on the aged myocardium and not the cells direct differentiation into cardiomyocytes.

REFERENCES:

1. El-Bialy A, Shenoda M, Saleh J, Tilkian A. Myocardial calcification as a rare cause of congestive heart failure: a case report. J Cardiovasc Pharmacol Ther. 2005;10(2):137-43.
2. Gowda RM, Boxt LM. Calcifications of the heart. Radiol Clin North Am. 2004;42(3):603-17, vi-vii.
3. Isaac C, Wright A, Usas A, Li H, Tang Y, Mu X, et al. Dystrophin and utrophin "double knockout" dystrophic mice exhibit a spectrum of degenerative musculoskeletal abnormalities. J Orthop Res. 2013;31(3):343-9.
4. Mu X, Usas A, Tang Y, Lu A, Wang B, Weiss K, et al. RhoA mediates defective stem cell function and heterotopic ossification in dystrophic muscle of mice. FASEB J. 2013;27(9):3619-31.
5. McBeath R, Pirone DM, Nelson CM, Bhadriraju K, Chen CS. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. Dev Cell. 2004;6(4):483-95.
6. Zhou H, Li YJ, Wang M, Zhang LH, Guo BY, Zhao ZS, et al. Involvement of RhoA/ROCK in myocardial fibrosis in a rat model of type 2 diabetes. Acta Pharmacol Sin. 2011;32(8):999-1008.
7. Castellani L, Salvati E, Alema S, Falcone G. Fine regulation of RhoA and Rock is required for skeletal muscle differentiation. J Biol Chem. 2006;281(22):15249-57.
8. Deconinck N, Dan B. Pathophysiology of duchenne muscular dystrophy: current hypotheses. Pediatr Neurol. 2007;36(1):1-7.
9. Towbin JA, Bowles NE. The failing heart. Nature. 2002;415(6868):227-33.
10. Marcus RL, Addison O, Kidde JP, Dibble LE, Lastayo PC. Skeletal muscle fat infiltration: impact of age, inactivity, and exercise. J Nutr Health Aging. 2010;14(5):362-6.
11. Cipriano CA, Pill SG, Keenan MA. Heterotopic ossification following traumatic brain injury and spinal cord injury. J Am Acad Orthop Surg. 2009;17(11):689-97.
12. Shi S, de Gorter DJ, Hoogaars WM, t Hoen PA, Ten Dijke P. Overactive bone morphogenetic protein signaling in heterotopic ossification and Duchenne muscular dystrophy. Cell Mol Life Sci. 2012.

13. Hannallah D, Peng H, Young B, Usas A, Gearhart B, Huard J. Retroviral delivery of Noggin inhibits the formation of heterotopic ossification induced by BMP-4, demineralized bone matrix, and trauma in an animal model. J Bone Joint Surg Am. 2004;86-A(1):80-91.
14. Miljkovic-Gacic I, Wang X, Kammerer CM, Gordon CL, Bunker CH, Kuller LH, et al. Fat infiltration in muscle: new evidence for familial clustering and associations with diabetes. Obesity (Silver Spring). 2008;16(8):1854-60. PMCID: 2895815.
15. Zoico E, Rossi A, Di Francesco V, Sepe A, Olioso D, Pizzini F, et al. Adipose tissue infiltration in skeletal muscle of healthy elderly men: relationships with body composition, insulin resistance, and inflammation at the systemic and tissue level. J Gerontol A Biol Sci Med Sci. 2010;65(3):295-9.
16. Corcoran MP, Lamon-Fava S, Fielding RA. Skeletal muscle lipid deposition and insulin resistance: effect of dietary fatty acids and exercise. Am J Clin Nutr. 2007;85(3):662-77.
17. Savage DB, Petersen KF, Shulman GI. Disordered lipid metabolism and the pathogenesis of insulin resistance. Physiol Rev. 2007;87(2):507-20. PMCID: 2995548.
18. Laforet P, Vianey-Saban C. Disorders of muscle lipid metabolism: diagnostic and therapeutic challenges. Neuromuscul Disord. 2010;20(11):693-700.
19. Hulver MW, Dohm GL. The molecular mechanism linking muscle fat accumulation to insulin resistance. Proc Nutr Soc. 2004;63(2):375-80.
20. Schulze PC. Myocardial lipid accumulation and lipotoxicity in heart failure. J Lipid Res. 2009;50(11):2137-8. PMCID: 2759818.
21. Axelsen LN, Lademann JB, Petersen JS, Holstein-Rathlou NH, Ploug T, Prats C, et al. Cardiac and metabolic changes in long-term high fructose-fat fed rats with severe obesity and extensive intramyocardial lipid accumulation. Am J Physiol Regul Integr Comp Physiol. 2010;298(6):R1560-70.
22. Ruberg FL. Myocardial lipid accumulation in the diabetic heart. Circulation. 2007;116(10):1110-2.
23. Zhou YT, Grayburn P, Karim A, Shimabukuro M, Higa M, Baetens D, et al. Lipotoxic heart disease in obese rats: implications for human obesity. Proc Natl Acad Sci U S A. 2000;97(4):1784-9. PMCID: 26513.
24. Sharma S, Adrogue JV, Golfman L, Uray I, Lemm J, Youker K, et al. Intramyocardial lipid accumulation in the failing human heart resembles the lipotoxic rat heart. FASEB J. 2004;18(14):1692-700.
25. Saini-Chohan HK, Mitchell RW, Vaz FM, Zelinski T, Hatch GM. Delineating the role of alterations in lipid metabolism to the pathogenesis of inherited skeletal and cardiac muscle disorders: Thematic Review Series: Genetics of Human Lipid Diseases. J Lipid Res. 2012;53(1):4-27. PMCID: 3243479.
26. Tahallah N, Brunelle A, De La Porte S, Laprevote O. Lipid mapping in human dystrophic muscle by cluster-time-of-flight secondary ion mass spectrometry imaging. J Lipid Res. 2008;49(2):438-54. PMCID: 2438276.
27. Ridley AJ. Rho GTPases and cell migration. J Cell Sci. 2001;114(Pt 15):2713-22.
28. Luo L. Rho GTPases in neuronal morphogenesis. Nat Rev Neurosci. 2000;1(3):173-80.
29. Khatiwala CB, Kim PD, Peyton SR, Putnam AJ. ECM compliance regulates osteogenesis by influencing MAPK signaling downstream of RhoA and ROCK. J Bone Miner Res. 2009;24(5):886-98. PMCID: 2672206.
30. Meyers VE, Zayzafoon M, Douglas JT, McDonald JM. RhoA and cytoskeletal disruption mediate reduced osteoblastogenesis and enhanced adipogenesis of human mesenchymal stem cells in modeled microgravity. J Bone Miner Res. 2005;20(10):1858-66. PMCID: 1351020.
31. Hosoyama T, Ishiguro N, Yamanouchi K, Nishihara M. Degenerative muscle fiber accelerates adipogenesis of intramuscular cells via RhoA signaling pathway. Differentiation. 2009;77(4):350-9.
32. Santos A, Bakker AD, de Blieck-Hogervorst JM, Klein-Nulend J. WNT5A induces osteogenic differentiation of human adipose stem cells via rho-associated kinase ROCK. Cytotherapy. 2010;12(7):924-32.
33. Goto K, Chiba Y, Sakai H, Misawa M. Tumor necrosis factor-alpha (TNF-alpha) induces upregulation of RhoA via NF-kappaB activation in cultured human bronchial smooth muscle cells. J Pharmacol Sci. 2009;110(4):437-44.
34. Slice LW, Bui L, Mak C, Walsh JH. Differential regulation of COX-2 transcription by Ras- and Rho-family of GTPases. Biochem Biophys Res Commun. 2000;276(2):406-10.
35. Charrasse S, Comunale F, Grumbach Y, Poulat F, Blangy A, Gauthier-Rouviere C. RhoA GTPase regulates M-cadherin activity and myoblast fusion. Mol Biol Cell. 2006;17(2):749-59. PMCID: 1356585.

36. Beqaj S, Jakkaraju S, Mattingly RR, Pan D, Schuger L. High RhoA activity maintains the undifferentiated mesenchymal cell phenotype, whereas RhoA down-regulation by laminin-2 induces smooth muscle myogenesis. J Cell Biol. 2002;156(5):893-903. PMCID: 2173321.
37. Lindegaard ML, Nielsen LB. Maternal diabetes causes coordinated down-regulation of genes involved with lipid metabolism in the murine fetal heart. Metabolism. 2008;57(6):766-73.
38. Lehman JJ, Kelly DP. Transcriptional activation of energy metabolic switches in the developing and hypertrophied heart. Clin Exp Pharmacol Physiol. 2002;29(4):339-45.
39. Okada M, Payne TR, Zheng B, Oshima H, Momoi N, Tobita K, et al. Myogenic endothelial cells purified from human skeletal muscle improve cardiac function after transplantation into infarcted myocardium. Journal of the American College of Cardiology. 2008;52(23):1869-80. PMCID: 2719893.
40. Crisan M, Yap S, Casteilla L, Chen CW, Corselli M, Park TS, et al. A perivascular origin for mesenchymal stem cells in multiple human organs. Cell stem cell. 2008;3(3):301-13.
41. Chen CW, Okada M, Proto JD, Gao X, Sekiya N, Beckman SA, et al. Human pericytes for ischemic heart repair. Stem Cells. 2012.
42. Okada M, Payne TR, Drowley L, Jankowski RJ, Momoi N, Beckman S, et al. Human skeletal muscle cells with a slow adhesion rate after isolation and an enhanced stress resistance improve function of ischemic hearts. Molecular therapy : the journal of the American Society of Gene Therapy. 2012;20(1):138-45. PMCID: 3255579.

Sub-project 2: Human hepatocytes for treatment of life-threatening liver injury
PI's: Ira Fox, MD and David Perlmutter, MD

INTRODUCTION: (New data is underlined in the text of the Body of the report.)
These studies are focused on expanding human hepatocytes from control, marginal quality and cirrhotic livers for the treatment of life-threatening acute liver failure. Two technical objectives were proposed: 1) to characterize and expand hepatocytes from patients with cirrhosis and end-stage liver disease in immune deficient hosts whose livers permit extensive repopulation with donor cells, and 2) to determine the extent to which transplantation with human hepatocytes can reverse hepatic failure in a clinically relevant non-human primate model of this process. In order to accomplish these objectives, we continue to explore the range of liver diseases that allow expansion of human hepatocytes in FRG mice and have isolated the human hepatocytes for use in a non-human primate model of acute liver failure. We have also performed additional studies on hepatocytes isolated from the livers of rats with end-stage cirrhosis, identified a target molecule that controls liver-specific gene expression in these cells and demonstrated that re-expression of this gene, HNF4□, results in normalization of hepatocyte function in vitro and in vivo. We have also induced acute liver failure in monkeys and transplanted these animals with human hepatocytes. While we were unsuccessful in correcting liver failure in the first animal transplanted with human hepatocytes, we have made progress in optimizing the protocol for inducing acute liver failure. We used this optimized protocol in a second transplant experiment, however, the animal died from a technical complication before the efficacy of transplantation could be assessed. When personnel issues are resolved, experiments will continue. We have also demonstrated that we can recover an adequate number of human hepatocytes from repopulated FRG mice for transplantation in a primate model.

Body:
Technical Objective #1: To characterize and expand hepatocytes from patients with cirrhosis and end-stage liver disease in immune deficient hosts whose livers permit extensive repopulation with donor cells.
Hypothesis: *Human hepatocytes derived from poor quality human cadaver donors can be resuscitated and expand in numbers that can be used for clinical application in the livers of immune deficient hosts where there is a selective repopulation advantage to transplanted donor hepatocytes.*

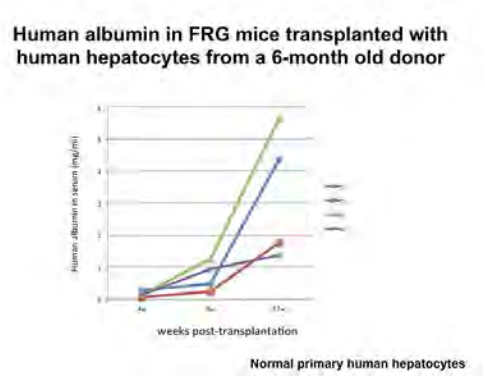


Figure 1

1.1. Expanding human hepatocytes in FRG mice.
We have performed primary transplants using human hepatocytes from non-cirrhotic donors as a source of cells for Technical Objective #2 in FRG mice. Human hepatocytes from the explanted liver of two patients with ornithine transcarbamylase (OTC) deficiency were transplanted into immune-deficient mice with hereditary tyrosinemia (FAH-/-; FRG). The level of human serum albumin (HSA) in the peripheral blood of all animals was greater than 1.5mg/ml, indicating at least 20% of the liver was replaced with human hepatocytes. One recipient animal was sacrificed, and approximately 50% engraftment was confirmed by immunohistochemistry. We then isolated hepatocytes from the remaining repopulated FRG mice and secondary transplants were performed with the recovered cells in 5 naïve FRG mice. The HSA levels in the transplanted mice were detectable 4 weeks after transplant, with a mean HSA level of 6.87 ± 0.91 ug/ml. This level of re-population, at this time point, was as expected based on the literature.

The time course of repopulation following transplantation using control hepatocytes in FRG mice is demonstrated in **Figure 1**. In addition, we have transplanted FRG mice using human hepatocytes derived from liver resection specimens from patients with metastatic colon cancer that have received cancer chemotherapy. The HSA levels in the mice transplanted with hepatocytes from patients receiving one and 6

cycles of chemotherapy are shown in **Figures 2 and 3**. Full repopulation (based on HSA level of from 1-9 mg/ml) can be seen following transplantation with hepatocytes from patients receiving one cycle of chemotherapy. The repopulation is not as strong from the patient that received 6-cycles of chemotherapy, but greater than 20% repopulation is seen, based on HSA levels greater than 1 mg/ml. The rate of repopulation was not affected by exposure to chemotherapy.

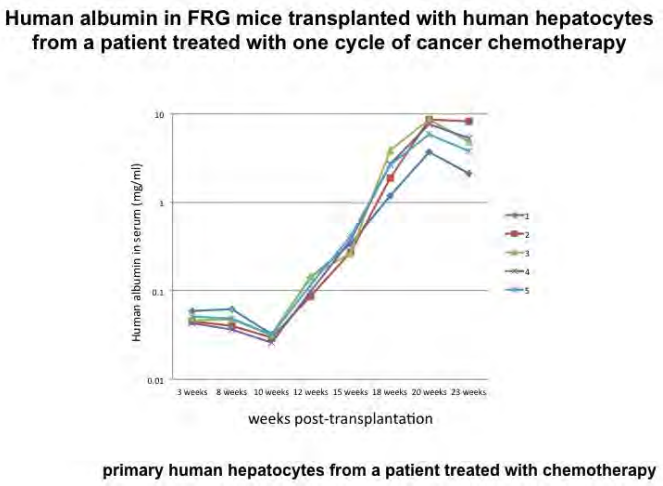


Figure 2

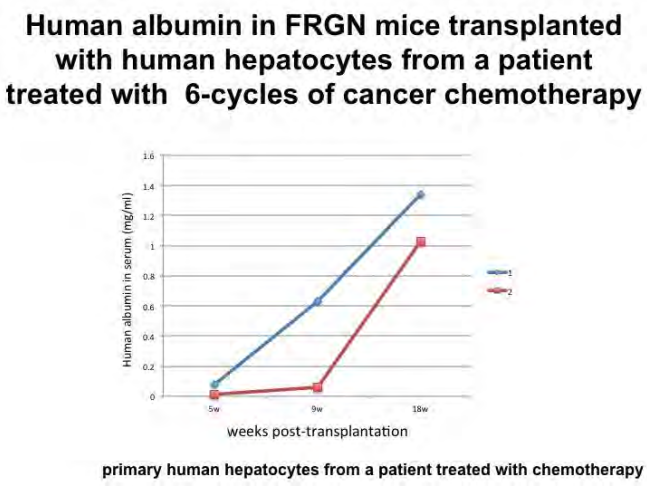


Figure 3

We have also successfully transplanted hepatocytes from four patients with cirrhosis. The diseases included alpha-1-antitrypsin deficiency, progressive familial intrahepatic cholestasis type 2, and Wilson's disease. As seen in **Figures 4-7**, engraftment and expansion of cells has been slower than that seen when hepatocytes from non-cirrhotic patients are transplanted, but high repopulation (based on HSA level of greater than 1mg/ml) could be seen in some of the transplanted mice (**Figure 5** and **Figure 7**), indicating that the primary human hepatocytes from patients with cirrhosis can be resuscitated and expand in FRG/FRGN mice.

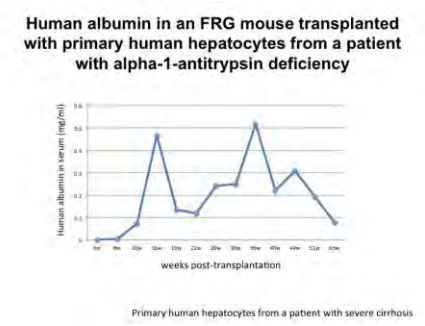


Figure 4

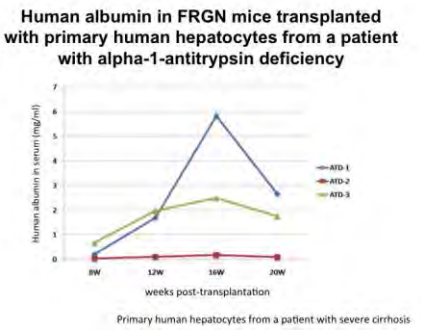


Figure 5

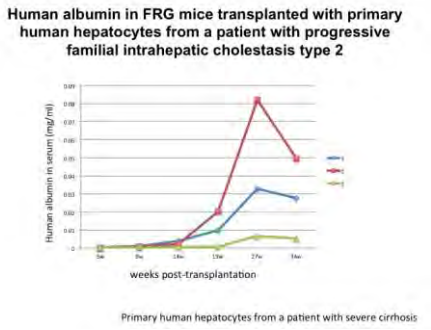


Figure 6

Analysis of tissue samples from one of the FRGN mice highly repopulated with primary human hepatocytes from a patient with alpha-1-antitrypsin deficiency (ATD) showed that the transplanted human hepatocytes incorporated into the hepatic parenchyma (**Figure 8**) and displayed PAS-positive/diastase-resistant globules that characterizes ATD (**Figure 9**).

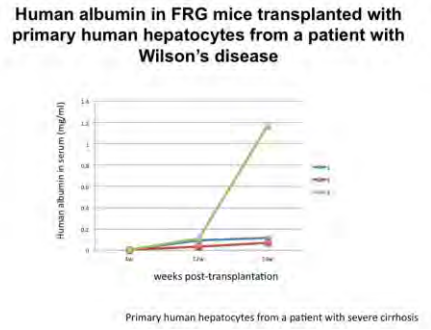


Figure 7

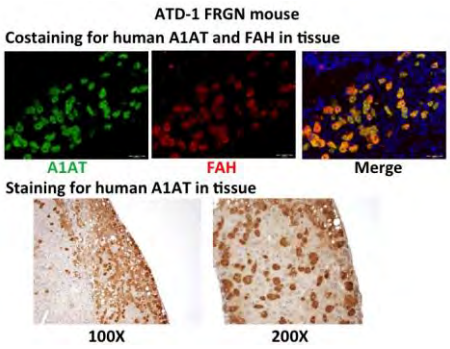


Figure 8

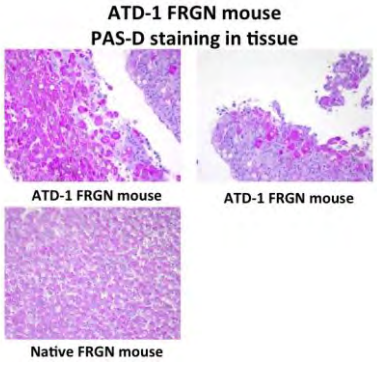


Figure 9

Tissue specimens and isolated hepatocytes from this FRGN mouse confirmed approximately 20% hepatic repopulation by immunostainings (**Figure 10**). Collagen deposition was detected by Picrosirius red staining within the liver parenchyma (**Figure 11**).

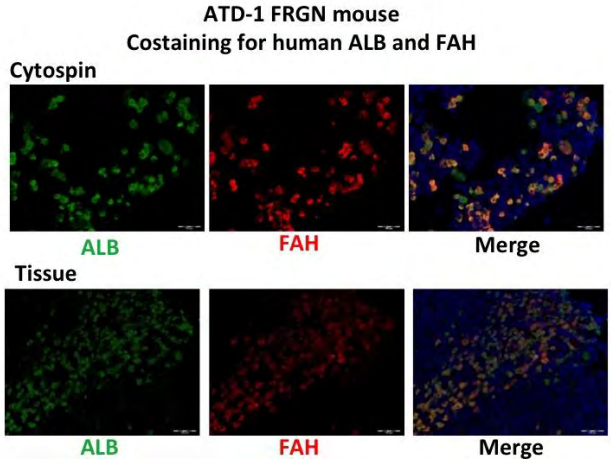


Figure 10

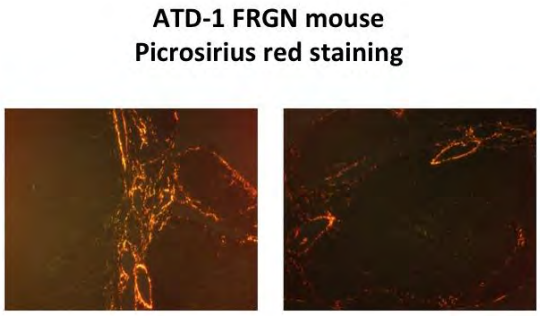


Figure 11

Pulse chase analysis of alpha-1-antitrypsin (AT) in the ATD hepatocytes isolated from the FRGN mouse revealed strikingly similar kinetics of degradation and secretion of AT compared to ATD hepatocytes recovered directly from the patient (analyzed prior to transplantation), indicating that the transplanted human cells retained their "disease characteristics" even after transplantation (**Figure 12**). Secondary transplants were also performed with the recovered cells in 4 naïve FRGN mice. These experiments are ongoing. In the future, we intend to transplant cells from cirrhotic livers that do not have a genetic abnormality (such as from patients with Laennec's cirrhosis or from patients with steatohepatitis) to determine whether such cells can repopulate FRG mouse livers more completely.

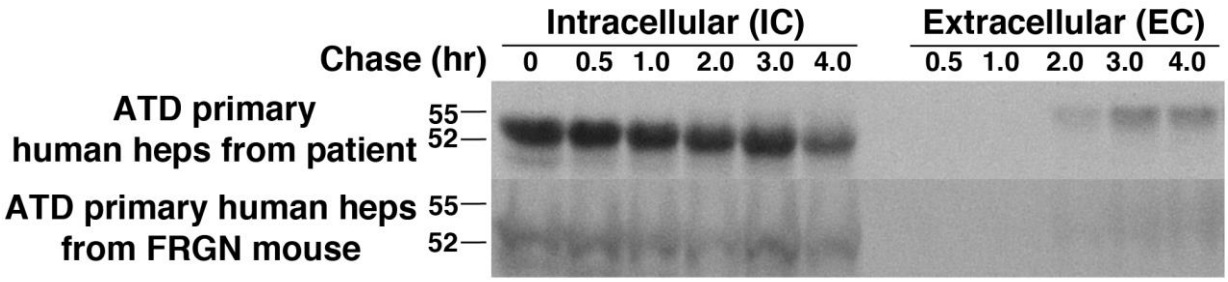


Figure 12

We eventually intend to transplant and repopulate additional immune deficient mice to generate cells for the

non-human primate acute liver failure studies outlined in Technical Objective #2.

1.2. Normalization of end-stage decompensated hepatocyte function in vitro and in vivo by re-expression of HNF4α.

In a continuation of studies to determine the extent to which hepatocytes derived from livers with severe chronic injury could be resuscitated for use in clinical hepatocyte transplantation, we isolated hepatocytes from the livers of Lewis rats with compensated and end-stage decompensated cirrhosis. To assess the extent to which hepatocyte-specific characteristics are affected by cirrhosis and liver failure, mRNA from isolated hepatocytes derived from cirrhotic and control livers were compared for gene expression by microarray analysis. As noted previously, hierarchical cluster analysis demonstrated significant gene expression differences among groups depending on the extent of cirrhosis from which the hepatocytes were derived. As expected, there were progressive changes in the expression of genes representing signals promoting proliferation and regeneration, apoptosis, and cell-death, most likely mediated by inflammation and oxidative stress, and progressive loss of gene expression representing worsening of metabolic function. This work has now been published (**Liu, et al. The microenvironment in hepatocyte regeneration and function in rats with advanced cirrhosis. Hepatology 2012; 55(5): 1529-39**). Microarrays also showed marked decreases in the expression of HNF4α, Foxa2, C/EBPα, and HNF1α, DNA binding proteins that are part of the network of hepatocyte-enriched transcription factors, sequentially established during development, that regulate the mature hepatocyte phenotype, controlling expression of proteins of coagulation, biliary metabolism, and lipid metabolism.

Since transcription factor deficiency could explain hepatocyte impairment, we investigated the therapeutic effects of forced re-expression. HNF4α was chosen for this therapy because it is the central regulator of the adult hepatocyte transcription factor network, has no other hepatocyte-expressed homolog, and showed the greatest reduction in the decompensated hepatocyte. We therefore performed a detailed analysis of the expression of HNF4α and its target genes in isolated hepatocytes and liver tissue. qRT-PCR analysis confirmed severe downregulation of HNF4α expression, and quantification of HNF4α in hepatocytes by western blot and by immunofluorescent staining of cytospin samples gave similar results. Thus, a significant decrease of HNF-4α in hepatocytes correlated with decompensation in cirrhosis.

To assess whether forced re-expression of HNF4α could affect the function of cirrhotic hepatocytes, we first used an *in vitro* culture system. Hepatocytes, isolated from animals with cirrhosis and decompensated liver function, were transduced with adeno-associated virus (AAV) vectors to express HNF4α and GFP or GFP alone. At 48 hours, qRT-PCR analysis showed HNF4α re-expression restored to nearly normal levels the network transcription factors C/EBPα, HNF1α, and PPARα, and the phenotypic target genes important for liver-specific activity. HNF4α expression also improved secretion of albumin into the culture supernatant—severely impaired in hepatocytes isolated from decompensated cirrhosis —and activity of Cytochrome P450 3A4, a major enzyme of xenobiotic metabolism. Animals with liver failure and cirrhosis were then transduced to re-express HNF4□ in their hepatocytes by intravenous infusion of $3x10^{11}$ AAV-HNF4α-GFP genomes. Animals sacrificed two weeks after infusion demonstrated high transduction efficiency uniformly distributed in most hepatocytes. Moreover, the impaired albumin expression of decompensated cirrhosis was dramatically improved and its expression increased until the time of sacrifice at 100 days following AAV treatment. Administration of the AAV-GFP control vector did not affect liver function. Finally, pathophysiologic testing showed striking and persistent improvement in liver function, ascites, activity, and neurologic function, and survival was prolonged to the end-point of the study at 100 days post AAV treatment. Functional analysis of cells isolated from treated animals showed significant improvement of albumin secretion and CYP3A4 activity. In addition, there was improvement in expression levels of HNF4α target genes and decreased expression of the hepatic progenitor cell markers AFP, CD44, and EpCAM**.** The healing effects of HNF4α re-expression did not depend on proliferation, since there was no increase apparent in Ki67 staining**.** HNF4α did not significantly augment TERT expression and telomere length in the cirrhotic hepatocytes remained critically

short**.** Thus, HNF4α acted by phenotypically correcting diseased hepatoctyes, not by stimulating their replacement.

These studies show that down-regulation of HNF4α has a profound effect on the end-stage cirrhotic hepatocyte in vitro, since replenishment of this single factor immediately revitalizes function. Moreover, transduction of hepatocytes in cirrhotic animals with apparently irreversible decompensated function produced a profound and immediate improvement in hepatic function. Normalization of function took place in two weeks. It is likely that cytokine/injury effects alter expression of the hepatocyte transcription factor network by extrinsic mechanisms, with the result that network factors establish a new steady-state equilibrium in the dysfunctional hepatocyte that can no longer compensate to restore normal gene expression. This possibility has important therapeutic implications, because it may require only transient therapy with HNF4α to restore the transcription factor network once the injury has been moderated. These studies suggest that in addition to regeneration mediated by expansion of mature hepatocytes or differentiation and expansion of induced progenitors, normalized function can be accomplished by transcriptional reprograming with reversal of de-differentiation but not senescence. The results also suggest HNF4α therapy could be effective in treating advanced liver cirrhosis with impaired hepatic function as a bridge to organ transplantation or possibly even as destination therapy.

These data have now been confirmed by RNA-Seq analysis (**Figure 13**). **Furthermore,** we have now shown that re-programming the liver-enriched transcription factor network by HNF4α-treatment did not require prolonged expression of virus-mediated HNF4α. Fourteen weeks after intervention essentially all HNF4α expression was mediated by the endogenous gene, and AAV-HNF4-specific transcripts were expressed at extremely low levels as shown by qPCR and RNA-seq (**Figure 14).** This lack of necessity for prolonged expression of exogenous reprogramming genes is similar to that seen following re-programming somatic cells to pluripotency. HNF4α is an essential transcription factor during embryonic development and its expression continues in the adult liver and in other extra-hepatic tissues. Since we did not examine whether AAV intervention resulted in enhanced extra-hepatic HNF4α expression, we cannot rule out an independent effect on a small number progenitor cells, but the restoration of the liver phenotype must occur by reprogramming and correction of hepatocytes.

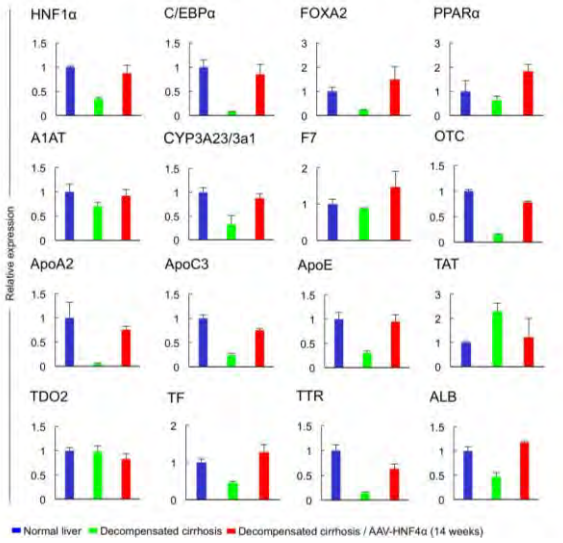


Figure 13

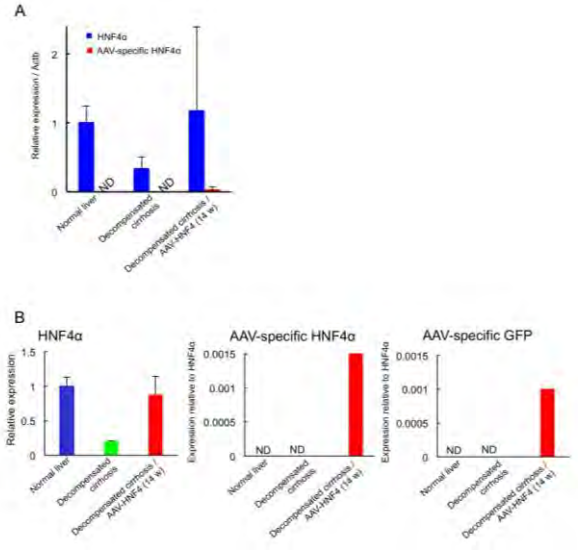


Figure 14

In addition, our studies now show that reversal of the distorted extracellular matrix is not absolutely required to reverse hepatic failure in degenerative liver disease as only minimal resolution of fibrosis was found by histology two weeks after HNF4α forced re-expression, well after improvement in hepatic function was documented (**Figure 15**). Significant improvement in histology, however, was observed at 100 days. These results are in sharp contrast to our previously published experience following hepatocyte transplantation. These

data are now in a manuscript under review after revision in the Journal of Clinical Investigation **(Nishikawa T, Bell A, Brooks JM, Setoyama K, Melis M, Han B, Fukumitsu K, Handa K, Tian J, Kaestner KH, Vodovotz Y, Locker J, Soto-Gutierrez A, Fox IJ. Resetting the transcription factor network reverses terminal chronic hepatic failure, JCI in revision).**

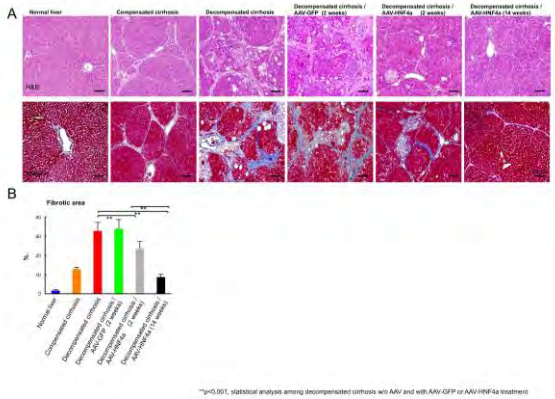


Figure 16

Technical Objective #2: To determine the extent to which transplantation with human hepatocytes can reverse hepatic failure in a clinically relevant non-human primate model of this process.
Hypothesis: *Human hepatocytes derived from human cadaver donors or possibly from human stem cells can reverse hepatic failure.*

2.1. Acute hepatic failure in a non-human primate model.

We have treated two additional non-human primates (NHP) with whole liver radiation therapy followed by total parenteral nutrition (TPN) in preparation for transplantation studies. The work has now been incorporated in a manuscript that is now in press (**Yannam GR, et al. A nonhuman primate model of human radiation-induced venocclusive liver disease and hepatocyte injury. Int. Journal of Radiation Oncology, Biology, Physics. 2014; 88(2): 404-11**).

Unfortunately, there has been a small setback in the ability to complete the transplant studies. The first NHP was irradiated with a dose of 40Gy to the whole liver. For logistical reasons, we delayed instituting TPN to induce acute liver failure. This has not been an issue in the past. In this case, however, it resulted in generating unanticipated non-lethal radiation induced liver disease (RILD) in the animal, which altered the architecture and vascular structure of the liver. We isolated human hepatocytes from several repopulated FRG mice to transplant into this animal, as outlined in the grant proposal. Because of the altered vascular structure we visualized severe shunting during the course of the hepatocyte transplant procedure by contrast imaging. Thus, no cells could be engrafted. The animal was electively euthanized and the liver histology confirmed the RILD and failure to engraft cells because of the altered vascular architecture. A dose of 35Gy to the whole liver was used on a second NHP. This dose was successful and the second animal was electively euthanized when we had determined that the RT dose was effective at ultimately inducing acute liver failure but did not lead to RILD.

Once the issue had been resolved we proceeded with an additional NHP transplant experiment for the treatment of acute liver failure. An animal was irradiated with a dose of 35Gy to the whole liver. The monkey was then transplanted through the inferior mesenteric vein with one billion hepatocytes. After the transplant, the 18G catheter was pulled and the mesenteric vein was ligated. The monkey recovered from the surgery, but his condition deteriorated afterwards in the first 24 hours. At autopsy, massive blood accumulation was found in the abdomen of this animal. It appeared that the bleeding was from the inferior mesenteric vein, the site where cells were infused. Since this was a serious technical problem, a number of protocol issues were

modified and team assignments were changed. Unfortunately, there was too much change in personnel after the procedure, and the senior technician in the lab with experience in NHP research left for a more senior position at Yerkes National Primate Center in Atlanta. When adequate personnel are available we will perform additional monkey transplant studies.

KEY RESEARCH ACCOMPLISHMENTS:

1. Engraftment and proliferation of human hepatocytes in immune-deficient FAH k/o transgenic (FRG) mice. Data supports our hypothesis that excellent quality human hepatocytes can be recovered from patients treated with chemotherapy and with end-stage cirrhosis. The transplanted human hepatocytes can retain their "disease characteristics" even after repopulation in the mice.
2. Identification of a key transcription regulator of hepatocyte function in end-stage decompensated hepatocytes from cirrhotic livers.
3. Demonstration that re-expression of HNF4 in decompensated cirrhotic hepatocytes leads to normalization of function in vitro and in vivo, and that this process does not require ongoing expression of the exogenous introduced gene. Ongoing studies, not shown, are examining whether this finding applies to human livers with hepatic failure.
4. Optimization of the non-human primate model of acute liver failure.
5. Isolation of an adequate supply of human hepatocytes from repopulated FRG mice for transplantation in NHP with acute liver failure.

REPORTABLE OUTCOMES:

1. Setoyama K, Fong JV, Han B, Ito R, Nagaya M, Ross M, Fukumitsu K, Gramignoli R, Rosensteel S, Strom SC, Stolz DB, Quader MA,Deutsch M, Baskin KM, Roy-Chowdhury J, Guha C, Soto-Gutierrez A, Fox IJ. 10-15% donor cell liver repopulation in non-human primates by low dose directed (right lobe) radiation therapy: a preclinical study. Hepatology 2011; 54(S1): 172A.
2 Liu,L, Yannam GR, Nishikawa T, Yamamoto T, Basma H, Ito R, Nagaya M, Dutta-Moscato J, Stolz DB, Duan F, Kaestner KH,Vodovotz Y, Soto-Gutierrez A, Fox IJ. The microenvironment in hepatocyte regeneration and function in rats with advanced cirrhosis. Hepatology 2012; 55(5): 1529-39.
3. Zhou H, Dong X, Kabarriti R, Chen Y, Avsar Y, Wang X, Ding J, Liu L, Fox IJ, Roy-Chowdhury J, Roy-Chowdhury N, Guha C. Single liver lobe repopulation with wildtype hepatocytes using regional hepatic irradiation cures jaundice in Gunn rats. PLoS One 2012; 7(10) :e46775.
4. Nishikawa T, Bell A, Brooks JM, Setoyama K, Kaestner KH, Vodovotz Y, Locker J, Soto-Gutierrez A, Fox IJ. Resetting the transcription network using a single factor reverses liver failure in end-stage cirrhosis. Journal of Clinical Investigation (In revision).
5. Yannam GR, Han B, Setoyama K, Yamamoto T, Ito R, Brooks JM, Guzman-Lepe J, Galambos C, Fong JV, Deutsch M, Quader MA, Yamanouchi K, Mehta K, Soto-Gutierrez A, Roy-Chowdhury J, Locker J, Abe M, Enke CA, Baranowska-Kortylewicz J, Solberg TD, Guha C, Fox IJ. Tolerable limits to whole liver irradiation in non-human primates. Hepatology 2012; 56(S1): 972A-973A.
6. NishikawaT, Bellance N, Damm A, Soto-Gutierrez A, Fox IJ, Nagrath D. Changes in Glycolysis are an Important Mechanism for Maintaining Cell Survival in Hepatic Failure in Advanced Cirrhosis. Hepatology 2012; 56(S1): 785A-786A.
7. Taichiro Nishikawa; Jenna M. Brooks; Yoram Vodovotz; Alejandro Soto-Gutierrez; Aaron W. Bell; Ira J. Fox 1284. Rescue of hepatic function in rats with advanced cirrhosis and end-stage liver failure following delivery of HNF4a. Hepatology 2012; 56(S1): 800A-801A.
8. Gramignoli R, Tahan V, Dorko K, Skvorak KJ, Hansel MC, Zhao W, Venkataramanan R, Ellis EC, Jorns C, Ericzon BG, Rosenborg S, Kuiper R, Soltys KA, Mazariegos GV, Fox IJ, Wilson EM, Grompe M, Strom SC. New potential cell source for hepatocyte transplantation: discarded livers from metabolic

disease liver transplants. Stem Cell Res. 2013 Jul;11(1):563-73.
9. Platt JL, Setoyama K, Fox IJ, "Xenotransplantation and the liver" In: Busuttil RW, Klintmalm GB, eds. Transplantation of the liver. W.B. Saunders Company; 3rd edition (October 1, 2013)
10. Fox IJ, Duncan SA. Engineering Liver Tissue from Induced Pluripotent Stem Cells: A first step in generating new organs for transplantation? Hepatology 2013;58(6):2198-2201.
11. Wheeler SE, Borenstein JT, Clark AM, Ebrahimkhani MR, Fox IJ, Griffith L, Inman W, Lauffenburger D, Nguyen T, Pillai VC, Prantil-Baun R, Stolz DB, Taylor D, Ulrich T, Venkataramanan R, Wells A, Young C. All-human microphysical model of metastasis therapy. Stem Cell Res Ther. 2013; 4 Suppl 1: S11.
12. Yannam R, Han B, Setoyama K, Yamamoto T, Ito R, Brooks JM, Guzman-Lepe J, Galambos C, Fong JV, Deutsch M, Quader MA, Yamanouchi K, Mehta K, Soto-Gutierrez A, Roy-Chowdhury J, Locker J, Abe M, Enke CA, Baranowska-Kortylewicz J, Solberg TB, Guha C, Fox IJ. A nonhuman primate model of human radiation-induced venocclusive liver disease and hepatocyte injury. (Int. Journal of Radiation Oncology, Biology, Physics. 2014; 88(2): 404-11).
13. Gramignoli R, Dorko K, Tahan V, Skvorak KJ, Ellis E, Jorns C, Ericzon BG, Fox IJ, Strom SC. Hypothermic storage of human hepatocytes for transplantation. Cell Transplant. 2014; 23(9):1143-51.
14. Hansel MC, Gramignoli R, Blake W, Davila J, Skvorak K, Dorko K, Tahan V, Lee BR, Tafaleng E, Guzman-Lepe J, Soto-Gutierrez A, Fox IJ, Strom SC. Increased Reprogramming of Human Fetal Hepatocytes Compared With Adult Hepatocytes in Feeder-Free Conditions. Cell Transplantation 2014; 23(1):27-38.
15. Mazariegos G, Shneider B, Burton B, Fox IJ, Hadzic N, Kishnani P, Morton DH, McIntire S, Sokol RJ, Summar M, White D, Chavanon V, Vockley J. Liver transplantation for pediatric metabolic disease. Mol Genet Metab. 2014;111(4):418-27.
16. Nishikawa T, Bellance N, Damm A, Han B, Zhu Z, Pipinos II, Soto-Gutierrez A, Fox IJ, Nagrath D. Failure of compensation mediated by glycolysis results in hepatocyte failure. Journal of Hepatology 2014;60(6):1203-11.
17. Chen Y, Chang C-J, Li Y, Ding J, Atienza K, Wang X, Avsar Y, Tafaleng E, Strom S, Guha C, Bouhassira EE, Fox IJ, Roy-Chowdhury J, Roy-Chowdhury N. Amelioration of hyperbilirubinemia in Gunn rats after transplantation of human induced pluripotent stem cell-derived hepatocytes. Stem Cell Reports (in revision).
18. Bhatia SN, Underhill GH, Zaret KS, Fox IJ. Cell and tissue engineering for liver disease. Science Translational Medicine 2014;6:245sr2.
19. Hayashi C, Ito M, Ito R, Murakumo A, Yamamoto N, Hiramatsu N, Fox IJ, Horiguchi A. Effects of edaravone, a radical scavenger, on hepatocyte transplantation. J Hepatobiliary Pancreat Sci. 2014 Sep 10. [Epub ahead of print]
20. Dutta-Moscato J, Solovyev A, Mi Q, Nishikawa T, Soto-Gutierrez A, Fox IJ, Vodovotz Y. A Multiscale Agent-Based in silico Model of Liver Fibrosis Progression. Front Bioeng Biotechnol. 2014; 2: 18.
21. Fox IJ, Daley GQ, Goldman SA, Huard J, Kamp TJ, Trucco M. Stem cell therapy. Use of differentiated pluripotent stem cells as replacement therapy for treating disease. Science. 2014 Aug 22;345(6199):1247391.
22. Dreyzin A, Barnato AE, Soltys KA, Farris C, Sada R, Haberman K, Fox IJ. Parent perspectives on decisions to participate in a phase I hepatocyte transplant trial. Pediatr Transplant. 2014; 18(1): 112-9.
23. Gramignoli R, Tahan V, Dorko K, Venkataramanan R, Fox IJ, Ellis EC, Vosough M, Strom SC. Rapid-and-Sensitive Assessment of Human Hepatocyte Functions. Cell Transplant. 2014 Apr 4. [Epub ahead of print]
24. Tafaleng, EN, Watson A, Fox IJ. "Normal Hepatocyte Function and Mechanisms of Dysfunction" In: Kleinman RE, Goulet O, Mieli-Vergani G, Sanderson IR, Sherman PM, Shneider BL, eds., Walker's Pediatric Gastrointestinal Disease: Physiology, Diagnosis, Management PMPH—USA. PMPH-USA (People's Medical Publishing House–USA. (in press)

25. Invited Speaker, Research Seminar Series in Developmental and Regenerative Biology, University of Kansas Medical Center, "Use of hepatocytes and stem cells to study and treat liver disease", Kansas City, Kansas, November 9-10, 2011.
26. Keynote Speaker, ISMRM Workshop on MRI-based cell tracking "Hepatocyte transplantation and the need to track engrafted cells", Miami Beach, Florida, January 29 – February 1, 2012.
27. Invited Speaker, American Society of Gene & Cell Therapy 15th Annual Meeting, "Overcoming barriers to successful cell therapy to treat liver disease", Philadelphia, PA May 16-19, 2012
28. Moderator, Mid-day Symposium: "Allotransplants, Cellular Transplants, Organ Repair, and Xenotransplants? A Debate about the Future of Organ Transplantation", American Transplant Congress, Boston, MA, June 2-6, 2012.
29. Invited Speaker, Liver Biology: Fundamental Mechanisms & Translational Application, FASEB Summer Research Conference, "Hepatocyte, stem cell transplantation, tissue engineering", Snowmass Village, Colorado, July 29 – August 3, 2012.
30. Invited Speaker, 8th Royan International Congress on Stem Cell Biology and Technology, "Overcoming barriers to the use of hepatocytes and stem cells in treating patients with liver diseases" and "Use of hepatocytes and stem cells in understanding and treating liver failure and cirrhosis", Tehran, Iran, September 5-7, 2012.
31. Invited Speaker, Masters of Surgery lecture series, Montefiore Medical Center, The University Hospital for Albert Einstein College of Medicine, "Bench to bedside: finding alternatives to organ transplantation for patients with life-threatening liver disease", New York, NY, November 4-5, 2012.
32. Faculty Member, American Association for the Study of Liver Diseases 2012 Postgraduate Course, "Tissue engineering and liver cell replacement – liver stem cells on the horizon", Boston, MA, November 10, 2012.
33. Invited Speaker, 23rd Conference of the Asian Pacific Association for the Study of the Liver (APASL 2013), "Future strategies for cellular transplantation", Singapore, June 6-10, 2013.
34. Invited Speaker, 19th Annual International Congress of ILTS, "Liver regeneration and hepatocyte repopulation", Sydney, Australia, June 14, 2013.
35. Invited Speaker, Cell Transplant Society, "Hepatocyte transplantation and regeneration in the treatment of liver disease", Milan, Italy, July 7-10, 2013.
36. Invited Speaker, AASLD/NASPGHAN Pediatric Symposium, The Liver Meeting 2013, "Pros and cons of hepatocyte transplantation for treatment of liver-based metabolic disease", Washington, DC, November 1, 2013.
37. Invited Speaker, Annual meeting – Adult Liver Stem Cell Transplantation Project, University Utrecht, "Bench to bedside: hepatocyte transplantation for the treatment of liver-based metabolic disease", Utrecht, The Netherlands, January 13, 2014.
38. Invited Speaker, Center for Cell and Gene Therapy Seminar Series, Baylor College of Medicine, "Bench to Bedside: Hepatocyte transplantation and regeneration in the treatment of liver disease", Houston, TX, February 4, 2014.
39. Invited Speaker, Whole Liver Replacement State-of-the-Science Summit, Chicago, IL, April 29-30, 2014
40. Invited Speaker, FASEB Summer Research Conference on Liver Biology: Fundamental Mechanisms & Translational Applications, "Modeling alpha-1-antitrypsin deficiency and PFIC using patient-derived pluripotent stem cells", Keystone, Co, July 10, 2014.
41. Invited Speaker, Bridging Biomedical Worlds conference: Turning Obstacles into Opportunities for Stem Cell Therapy, "Translating stem cells to the treatment of life-threatening liver disease." Beijing, China, October 13-15, 2014
42. Invited Speaker, Advances and Applications of Functional Hepatocytes (AAFH), "Treatment of life-threatening liver disease by primary or stem cell-derived hepatocyte transplantation." Shanghai, China, October 29-30, 2014

CONCLUSIONS:

As before, the outcomes of our studies are being accomplished.

Human muscle-derived stem cells prevent cardiac dysfunction in the aging dystrophic heart

William C.W. Chen[1,2], Xueqin Gao[1], Talgat Yessenov[1], Kimimasa Tobita[4], Arman Saparov[5], Aiping Lu[1], and Johnny Huard[1,*]

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Duchenne muscular dystrophy (DMD) is a recessive X-linked degenerative muscle disease that leads to progressive muscle weakness. The reduced level or lack of sarcolemma dystrophin in DMD patients affects not only skeletal muscles but also the myocardium, resulting in the development of dilated cardiomyopathy. Due to the extended life span of DMD patients, benefitted from new therapeutic modalities, a suitable treatment for DMD cardiac dysfunction has become increasingly important. Previously our group has demonstrated the regenerative potential of murine skeletal muscle-derived stem cells (mMDSCs) in the myocardium of mdx mice (a mouse DMD model). Here we investigate the therapeutic potential of human skeletal muscle-derived stem cells (hMDSCs) in the aging dystrophic heart where dilated cardiomyopathy gradually manifests. hMDSCs were isolated by a modified preplate technique from post-mortem human muscle biopsies. Cultured hMDSCs were transduced with lentiviral-GFP vectors and subsequently sorted by flow cytometry based on GFP expression. 1 x 10^6 GFP+ hMDSCs were intraperitoneally injected into the lower abdomen of aged immunodeficient dystrophic mice (mdx/SCID mice, 15-16 months old). The control group received PBS injections. Cardiac function was repeatedly assessed by echocardiography (N=6/group) immediately before and at 1 and 2 months following the injection. At 2 months post-injection, LV contractile function had dramatically deteriorated in control hearts. In sharp contrast, LV contractility was notably sustained following the hMDSC injection (Figure 1; LVFS, $p=0.006$; LVFAC, $p \leqq 0.001$; LVEF, $p \leqq 0.001$). Significant enlargement in LV chamber dimension was also recorded in control mice at 2 month post-injection, suggesting progressive LV dilatation. In hMDSC-injected mice, markedly smaller LV chamber

sizes were observed (Figure 1; LVEDA, p=0.011; LVESA, $p \leqq 0.001$), indicating prevention of adverse cardiac remodeling. Immunohistochemical analysis (N=4/group) revealed increased CD31+ capillary structures in the myocardium of hMDSC-injected mice (60.86±8.45/HPF) when compared with control mice (52.14±4.86/HPF). Similarly, more alpha-smooth muscle actin (αSMA)-positive vessels were observed in hMDSC-injected mice (4.57±0.89/HPF) when compared with control mice (3.86±0.65/HPF), suggesting the promotion of angiogenesis and vasculogenesis in the myocardium following intraperitoneal hMDSC injection. Preliminary analysis of myocardial fibrosis by Mason Trichrome staining showed reduced fibrotic area fraction in hMDSC-treated mice (N=2/group). Additionally, a very small number of GFP+ cells (<0.1%) could be detected in the myocardium, suggesting migration of implanted hMDSCs to the diseased heart. In conclusion, our data suggest a robust therapeutic potential of intraperitoneal hMDSC implantation in preventing cardiac dysfunction of aging dystrophic hearts. Currently we are investigating the terminal cell fate of implanted hMDSCs and the mechanistic pathway(s) involved in the hMDSC-mediated therapeutic effects imparted on aged dystrophic hearts.

Intraperitoneal administration of human muscle-derived stem cells reduce interstitial fibrosis, activate Wt1+ epicardial progenitor cells, and prevent cardiac dysfunction in the aging dystrophic heart

William C.W. Chen[1,2], Talgat Yessenov[1], Aiping Lu[1], Xueqin Gao[1], Kimimasa Tobita[4], Arman Saparov[5], and Johnny Huard[1,*]

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Duchenne muscular dystrophy (DMD), an inherited degenerative muscle disorder, affects not only skeletal muscles but also the myocardium. The reduced level or lack of sarcolemma dystrophin often leads to the development of dilated cardiomyopathy, especially in DMD patients with extended life spans. Consequently, therapies for DMD-associated cardiac dysfunction are urgently needed. Our group has previously demonstrated the regenerative potential of murine skeletal muscle-derived stem cells (mMDSCs) in the myocardium of mdx mice (a DMD model). Here we investigate the therapeutic potential of human skeletal muscle-derived stem cells (hMDSCs) in the aging dystrophic heart where dilated cardiomyopathy progressively manifests. hMDSCs were isolated from human muscle biopsies by a modified preplate technique. Cultured hMDSCs were transduced with lentiviral-GFP vectors and subsequently sorted by flow cytometry based on GFP expression. A single dose of one million GFP+ hMDSCs was intraperitoneally injected into the lower abdomen of aged mdx/SCID mice (15-16 months old). Control mice received PBS injections. At 2 month post-injection, while controls hearts exhibited extensive interstitial fibrosis within the myocardium (8.68±1.94%), hMDSC-treated hearts showed a 73.4% reduction of myocardial fibrosis (2.31±0.40%, p=0.018, N=4/group). hMDSC-treated group also showed prominent activation of Wt1+ epicardial progenitor cells (10.84±2.13 cells/HPF) when compared with control hearts (0.23±0.23 cells/HPF) (p=0.009, N=4/group). In addition, increased numbers of CD31+ capillaries (60.86±8.45/HPF) and alpha-smooth muscle actin (αSMA)-positive blood vessels (4.57±0.89/HPF) were recorded in the myocardium of hMDSC-injected mice when compared with the amount of CD31+ capillary (52.14±4.86/HPF) and αSMA+ vessel (3.86±0.65/HPF)

structures in control hearts (N=4/group). This suggests the promotion of angiogenesis and vasculogenesis in the myocardium following intraperitoneal hMDSC injection. Cardiac function was repeatedly assessed by echocardiography (N=6/group) immediately before and at 1 and 2 months following the injection. At 2 months post-injection, LV contractile function had dramatically deteriorated in control hearts but was notably sustained in hMDSC-treated hearts (LVFS, $p=0.006$; LVFAC, $p \leqq 0.001$; LVEF, $p \leqq 0.001$). Significant enlargement in LV chamber dimension was also documented in control mice at 2 months post-injection, suggesting progressive LV dilatation. In hMDSC-injected mice, markedly smaller LV chamber sizes were observed (LVEDA, $p=0.011$; LVESA, $p \leqq 0.001$), indicating prevention of adverse cardiac remodeling. Moreover, a very small number of GFP+ cells (<0.1%) could be detected in the myocardium, suggesting migration of implanted hMDSCs to the diseased heart. In conclusion, our data suggest a robust therapeutic potential of intraperitoneal hMDSC implantation in reducing interstitial fibrosis, activating Wt1+ epicardial progenitor cells, and preventing cardiac dysfunction in the dystrophic heart. Currently we are investigating the terminal cell fate of implanted hMDSCs and the mechanistic pathway(s) involved in hMDSC-mediated therapeutic effects imparted on aged dystrophic hearts.